Micro -algae biomass as an alternative resource for fishmeal and fish oil in the production of fish feed

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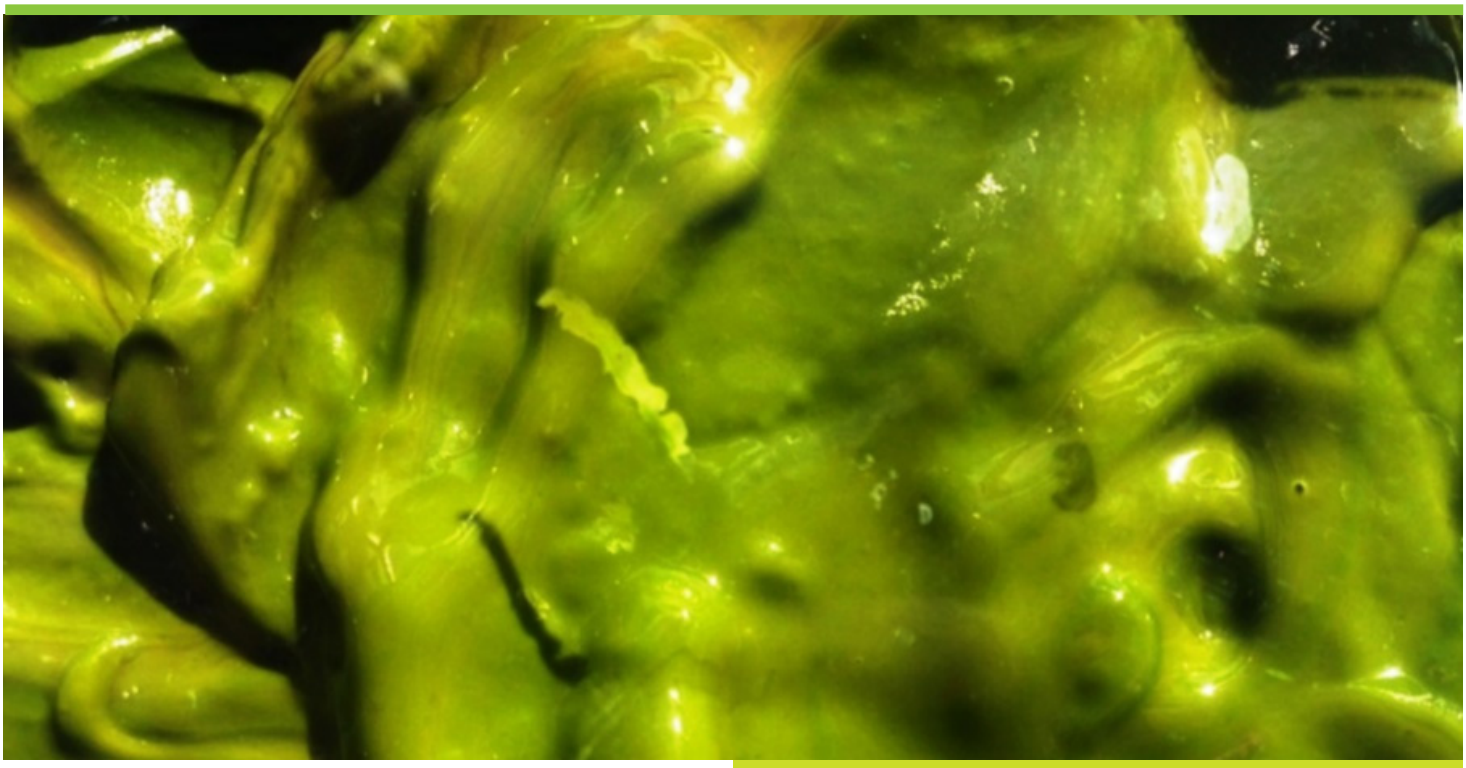

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Hamed Safafar
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DTU Food
National Food Institute

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PhD Thesis by Hamed Safafar

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Technical University of Denmark

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PREFACE

This PhD project entitled as "Micro -algae biomass as an alternative resource for fishmeal and fish oil in the production of fish feed" was carried out at the National Food Institute, Technical University of Denmark, under the supervision of Professor Charlotte Jacobsen as main supervisor (DTU-Food) and Dr. Per Møller as co-supervisor (Department of development, Kalundborg commune). The project was started in November 2013 and ended in November 2016.

This project was a part of a big project "Development of filtering technologies for microalgae and sustainable, high-quality feed for fry, (FIMAFY)" funded by Grønt Udviklings og Demonstrations Program (GUDP). The project partners include DTU-Aqua, BioMar A/S, LiqTech International A/S, IFAU and Ecolipids.

The PhD project aimed at developing new technologies for downstream processing of microalgae as a fish feed ingredient. The PhD project screened and selected the proper microalgae species, characterised the biochemical composition of algal biomass during the cultivation, processing and the storage and finally developed new technologies for downstream processing of microalgal biomass.

During the project, several algae-specific analytical methods were developed. Moreover, a microalgae cultivation laboratory including pilot scale downstream processing facilities (harvest, up-concentration, pasteurisation and drying) was established at DTU Food. I am deeply proud to see that young researchers are employing these methods and facilities for their research.

November 15, 2016

Kongens Lyngby, Denmark

Hamed Safafar

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After an intensive period of three years, today is the day: writing this note of thanks is the finishing touch on my thesis. First and foremost I wish to express my deepest gratitude to my supervisor Professor Charlotte Jacobsen for her continuous support, patience and motivation during the project. Your guidance helped me in all the time of research and writing of this thesis. I am heartily thankful to you. My sincere thanks also goes to Per who was a supportive co-supervisor. I learned a lot from both of you.

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My beloved family Maryam and Arian deserve a particular note of thanks. Maryam, your supports and patience during these years helped me to finish this project as it is now.

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Hamed Safafar

Summary

In recent years, intense efforts have been made to find new, alternate and sustainable aquatic feed ingredients, primarily in anticipation of an increasing world population and predicted insufficient fishmeal supply which is a critical component of aquaculture feed. Now it is becoming increasingly evident that the continued exploitation of industrial fish as a resource fish feed will ultimately become both environmentally and economically unsustainable. Microalgae are at the base of the entire aquatic food chain and play a major role in the diet of aquatic animals such as fish. Microalgae's main application for aquaculture are related to nutrition, being used as a sole fresh feed or an additive, e.g. source of pigment. Algae produce almost all nutritious compounds which are required for fish. The diverse biochemical composition of microalgae represents them as a promising candidate for the formulation of fish feed. The nutritional composition of microalgae depends on the species, environmental conditions and growth medium composition. Microalgae for use in aquaculture should be non-toxic and possess the essential nutritive constituents, in a reasonable price. Photosynthetic production of algae either in outdoor or indoor photobioreactor systems is costly since cultures must be maintained at low densities. Consequently, large volumes of media must be processed to recover small quantities of algae, and since most algal cells are minuscule, unspecific expensive harvesting processes must be employed. Strategies such as cultivation of microalgae on low price growth media, selection of microalgae capable of growing on such media and produce biomass with desired chemical composition and development of specific harvest and downstream processing represent basic solutions to improve the applicability of microalgae biomass as a fish feed ingredient. Moreover, storage of the algae biomass at optimum conditions minimise the deterioration of valuable compounds. This project has employed the strategies mentioned above to provide a clear concept for the cultivation, processing and the storage of microalgae biomass intended to be used as a fish feed ingredient. A pre-gasified industrial process water with high concentration of ammonia and free from toxic compounds, representing effluent from a local biogas plant was used as a low price growth medium. Therefore, the biomass production benefits from low cultivation price and also from valorization of the nutrients. Screening of various microalgae species has been extensively done to find proper microalgae capable of growing on industrial process water and producing a biomass containing high levels of protein, long-chain polyunsaturated fatty acids (LC PUFA), and bioactive compounds such as natural antioxidants. Effects of growth media composition/concentration and cultivation time on the nutritional composition of the biomass, variations in proteins, lipid, fatty acid composition, amino acids, tocopherols, and pigments were evaluated. Among all studied species including *Nannochloropsis salina, nannochloropsis limnetica, Chlorella sorokiniana, Chlorella vulgaris,*

Chlorella pyrenoidosa, Desmodesmus sp. and *Arthrospira platensis*, the microalgae *Chlorella pyrenoidosa* grew well on the industrial process water, efficiently valorized the compounds in the growth medium (ammonia and phosphorous) and produced reasonable amounts of the biomass (6.1 g/L). The resulting biomass included very high levels of protein (65.2±1.30% DW) as well as promising amino acid and carotenoid compositions. *Chlorella pyrenoidosa* was selected as a source of proteins and amino acids while lacking LC PUFA's. The microalgae *Nannochloropsis salina* which was grown on a mixture of standard growth medium and industrial process water produced a biomass containing high eicosapentaenoic acid (C20:5 n-3, as 44.2% ± 2.30% of total fatty acids), representing a rich source of LC PUFA. Data from laboratory scale experiments were translated to large scale and both of these species have been successfully cultivated in flat panel photobioreactor systems. Chromatographic methods were developed and employed for characterising algal biomass at both pre- and post-harvest stages and were based on the analysis of fatty acids (gas-liquid chromatography) and pigments (high-performance liquid chromatography). These methods represented rapid, routine and reliable control measures to verify the variations in the purity of the biomass the microalgae biomass during cultivation, and its quality during the processing and storage. In this study, a new downstream process set up, which included cross flow microfiltration by SiC (0.1μm) ceramic membranes, heat treatment (75℃&15 seconds) for inactivation of enzymes, up concentration by bowl centrifuge at 6500±500 g and finally drying by the novel swirl(spin) flash dryer was developed. This processing concept was specifically designed and tested on microalgae samples as a fish feed ingredient. The process aimed at reducing the energy consumption and minimizing deterioration of value-added bioactive compounds such a carotenoids, and LC PUFA. The method has been tested in the laboratory and large scales. Energy consumption per kg of the product was evaluated as 2.2 KWh, which was estimated as 28% lower than known current processing technologies which are being applied to microalgae. The swirl flash dryer was specifically designed to handle microalgae paste like feeds. Analysis of the pigment and fatty acid composition also revealed that the drying technique had profound adverse effects on the quality of microalgae biomass. As the final part of the study, effects of the storage time (0-56 days), storage temperature (5℃, 20℃ and 40℃) and the packaging conditions (under vacuum or ambient pressure) on a high LC PUFA biomass from *Nannochloropsis salina* was investigated. The storage time and temperature strongly influenced the oxidation reactions, which resulted in deterioration of bioactive compounds such as carotenoids, tocopherols and LC PUFA. The study revealed that the oxidation reactions, which led to the creation of primary and secondary products, occurred mainly during the first days of storage. The storage of freeze-dried microalgae at a low temperature (e.g. 5℃) was found to be more efficient than in oxygen-reduced storage conditions such as vacuum packaging.

This project provides imperative data covering all aspects of utilisation of algae biomass as a fish feed ingredient. These findings reveal new opportunities and open new doors toward research, processing and quality control in the microalgae industry.

OPSUMMERING

I de senere år har der været fokus på at finde nye, alternative og bæredygtige ingredienser til fiskefoder. Dette skyldes primært, at man forventer en kraftig befolkningstilvækst i verden samtidig med, at der vil være en utilstrækkelig produktion af fiskemel, som er en kritisk ingrediens i fiskefoder. Det bliver mere og mere klart, at en forsat udnyttelse af industrifisk som en ressource til fiskefoder ikke vil være økonomisk og miljømæssig bæredygtig. Mikroalger er det første led i den akvatiske fødekæde og spiller en vigtig rolle i akvatiske organismers (f.eks. fisks) diæt. Inden for akvakulturområdet finder mikroalger derfor anvendelse i ernæringen, enten som den eneste foderkilde (i uforarbejdet form) eller som additiv (f.eks. pigmenter). Mikroalger producerer næsten alle næringsstoffer, som fisk har behov for. Den biokemiske sammensætning af mikroalger gør dem til lovende kandidater til nye ingredienser i fiskefoder. Den ernæringsmæssige sammensætning af mikroalger afhænger af arten, vækstbetingelser og vækstmediets sammensætning. Mikroalger, som skal anvendes til akvakulturformål, skal være non-toksiske, indeholde alle næringsstoffer og skal kunne købes til en overkommelig pris. Produktion af mikroalger ved photosyntese, enten i udendørs eller indendørs photobioreaktorer er dyrt, fordi kulturen skal vedligeholdes i lav densitet. Som en konsekvens heraf er det nødvendigt at processere store voluminer algemedie for at opnå relativt små mængder algebiomasse. Næsten alle algeceller er meget små, hvilket indtil nu har resulteret i dyre høstprocesser. Det er derfor nødvendigt med strategiske løsninger, som kan resultere i dyrkning af alger i billige vækstmedier, udvælgelse af alger, som kan gro i sådanne medier og producere biomasse med den rigtige kemiske sammensætning, og udvikling af specifikke høst og "downstream-processer", der kan muliggøre anvendelse af mikroalger som ingrediens i fiskefoder til en overkommelig pris. Derudover er det vigtigt at opbevare den høstede algebiomasse ved optimale betingelser, således at nedbrydningen af værdifulde stoffer reduceres mest muligt.

Dette projekt har anvendt ovennævnte strategier med henblik på at udvikle et færdigt koncept til dyrkning, processering og opbevaring af mikroalge-biomasse, således at den kan anvendes som ingrediens i fiskefoder. Industrielt procesvand, som var blevet "afgasset" i et lokalt biogasanlæg, og som havde et højt indhold af ammoniak og var fri for toksiske stoffer, blev anvendt som et billigt vækstmedie. Biomasse-produktionen kunne derved drage fordel af lave udgifter til vækstmediet samtidig med at der skete en værdiforøgelse af næringsstofferne i procesvandet. Der blev foretaget en omfattende screening af forskellige mikroalge-arter med henblik på at finde de arter, som kunne gro på dette vækstmedie, og som kunne producere biomasse, som indeholdt høje niveauer af protein,

langkædede polyumættede fedtsyrer (LC PUFA) og bioaktive stoffer såsom naturlige antioxidanter. Effekten af vækstmediets sammensætning/koncentration og dyrkningstid på den ernæringsmæssige sammensætning af biomassen samt på variationer i protein- og lipidindhold, fedtsyre- og aminosyresammensætning, indhold af tocopheroler og pigmenter blev undersøgt. Blandt de undersøgte arter (*Nannochloropsis salina, Nannochloropsis limnetica, Chlorella sorokiniana, Chlorella vulgaris, Chlorella pyrenoidosa, Desmodesmus* sp. og *Arthrospira platensis*), voksede *Chlorella pyrenoidosa* godt i det industrielle procesvand, den tilførte værdi til vækstmediets indholdsstoffer (ammoniak og phosphor) og producerede rimelige mængder biomasse (6.1 g/L). Slutbiomassen havde et meget højt proteinindhold (65.2±1.30% tørvægt) og en lovende aminosyre- og carotenoidsammensætning. *Chlorella pyrenoidosa* blev derfor udvalgt som en kilde til protein og aminosyrer, men indeholdt dog ikke LC PUFA. Mikroalgen *Nannochloropsis salina,* som blev dyrket på en blanding af standard vækstmedium og industrielt procesvand producerede biomasse med et højt indhold af eicosapentaensyre (C20:5 n-3 med 44.2% ± 2.30% af total fedtsyreindholdet) og var dermed en rig kilde til LC PUFA. Data fra laboratorieforsøg blev overført til storskala, og begge arter er med succes blevet dyrket i "flat panel" photobioreaktorer. Kromatografiske metoder til karakterisering af alger både før og efter høst blev udviklet. Gas-væske-kromatografi (GLC) blev anvendt til analyse af fedtsyrerne og højtryksvæskekromatografi (HPLC) blev anvendt til analyse af pigmenter. Disse metoder er hurtige og kan anvendes rutinemæssigt til en pålidelig kontrolanalyse for at bekræfte variationer i renhed af biomassen under dyrkningen og for at bekræfte dens kvalitet under processering og opbevaring.

I dette PhD studium er der blevet udviklet et nyt downstream proceskoncept, som inkluderede cross flow mikrofiltrering med SiC (0.1µm) keramiske membraner, varmebehandling (75°C & 15 sekunder) til inaktivering af enzymer, opkoncentrering i en bovle centrifuge ved 6500±500 g og endelig tørring ved en ny "swirl (spin) flash tørrer". Dette proceskoncept blev specifikt designet og testet på mikroalge-prøver til brug som fiskefoder-ingrediens. Det blev tilstræbt, at processen skulle have et lavt energiforbrug og, at den skulle mimimere nedbrydningen af værdifulde bioaktive stoffer såsom carotenoider og LC PUFA. Metoden er blevet testet i laboratorie og stor skala. Energiforbruget pr kg produkt er beregnet til at være 2.2 KWh, som estimeres at være 28 % mindre en kendte procesteknologier, som i dag anvendes til mikroalger. Swirl flashtørreren blev specifikt designet til at håndtere mikroalge-pasta lignende indgangsmaterialer. Analyse af pigment- og fedtsyresammensætningen afslørede, at den sædvanlige anvendte tørreteknik (spraytørring) havde særdeles negative effekter på kvaliteten af mikroalge-biomassen, mens dette ikke var tilfældet for swirl flashtørreren.

I den sidste del af PhD studiet blev effekten af lagringstid (0-56 dage), lagringstemperatur (5 °C, 20 °C og 40 °C) samt emballeringsbetingelser (under vakuum eller atmosfærisk tryk) på en *Nannochloropsis salina* biomasse undersøgt. Lagringstid- og temperatur havde signifikant indflydelse på

oxidationsreaktionerne, hvilket resulterede i nedbrydning af bioaktive stoffer så som carotenoider, tocopheroler og LC PUFA. Undersøgelsen afslørede, at oxidationsreaktionerne, som førte til dannelsen af primære og sekundære oxidationsprodukter, primært fandt sted i de første dage af lagringsperioden. Opbevaring af frysetørret mikroalge-biomasse ved lav temperatur (f.eks. 5 ^{o}C) var mere effektivt i forhold til at forhindre oxidation end vakuumpakning.

Dette projekt har resulteret i vigtige resultater, som dækker alle aspekter vedrørende anvendelse af algebiomasse som ingrediens i fiskefoder. Resultaterne har afdækket nye muligheder og åbnet nye døre til forskning, processering og kvalitetskontrol i mikroalge-industrien.

LIST OF PUBLICATIONS

Paper 1 **Safafar, H.,** Van Wagenen, J., Møller, P., & Jacobsen, C. (2015), Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater. *Marine Drugs. 13*(12), 7339-7356; doi:10.3390/md13127069

Paper 2 **Safafar ,H.,** Møller, P., Jacobsen, C., Two-step direct transesterification as a rapid method for the analysis of fatty acids in microalgae biomass. Food Analytical Methods. submitted (November, 2016).

Paper 3 **Safafar, H.,** Z. Hass, M., Møller, P., L. Holdt, S., & Jacobsen, C. (2016). High-EPA Biomass from *Nannochloropsis salina* Cultivated in a Flat-Panel Photo-Bioreactor on a Process Water-Enriched Growth Medium. *Marine Drugs.* 14(8), 144; doi: 10.3390/md14080144

Paper 4 **Safafar ,H.,** Uldall Nørregaard, P., Ljubic, A., Løvstad Holdt, S., Møller, P., Jacobsen, C. Enhancement of protein and pigment content in two Chlorella sp. cultivated on industrial process water. *Journal of Marine Science and Engineering*. Submitted (September, 2016).

Paper 5 **Safafar ,H.,** Møller, P., Jacobsen, C. Downstream processing of microalgae with particular focus on its application as a fish feed ingredient. *Bioprocess and Biosystems Engineering.* Submitted (November, 2016).

Paper 6 **Safafar ,H.,** Langvad, S, Møller, P., Jacobsen, C. Storage conditions affect oxidative stability and nutritional composition of 3 freeze-dried *Nannochloropsis salina. European Journal of Lipid Science and Technology*. Submitted (November, 2016).

PATENT APPLICATIONS

EP 16201486.4. Safafar, H., Jacobsen, C., Møller, P., Asperud Reesbøll, C. Downstream processing & harvest of microalgae with a SiC membrane for production of food/feed ingredients.

EP 16201491 Safafar, H., Jacobsen, C., Møller, P., Asperud Reesbøll, C. Drying system of microalgae biomass with a swirling paddle.

OTHER PUBLICATIONS (not used in this Thesis)

Van Wagenen, J., Pape, M. L., **Safafar, H**., D'Este, M., DeFrancisci, D., Angeldaki, I. (2016) Biochemical profiles of waste-grown *Chlorella sorokiniana* in different process conditions. J. appl. Phycology. Submitted (July 2016).

Gringer, N.; **Safafar, H.**; Mesnildot, A.d.; Nielsen, H.H.; Rogowska-Wrzesinska, A.; Undeland,I.; Baron, C.P. (2015). Antioxidative low molecular weight compounds in marinated herring (*Clupea harengus*) salt brine. Food Chemistry. DOI: 10.1016/j.foodchem.2015.08.121

Naidenova, J., **Safafar, H.**, Jacobsen, C. Nutritional composition and antioxidative properties of some commercially produced microalgae powder. Prepared

Safafar, H., Ljubic, A., Uldall Nørregaard, P., Løvstad Holdt, S., Møller, P., Jacobsen, C. Variation of chemical compositions of microalgae *Chlorella minutissima* grown on process water. Prepared.

CONFERENCE ABSTRACTS AND PRESENTATIONS

Jacobsen, C., **Safafar, H.**, Langvad, S., & Møller, P. (2016). Oxidative Stability of High EPA, Freeze Dried *Nannochloropsis salina* in Relation to its Natural Antioxidant Composition. Ghent, Belgium.

Sørensen, A.-D. M., **Safafar, H**., Pedersen, A., Silva Marinho, G., & Holdt, S. L. (2016). Seasonal variations of antioxidants in the brown seaweed *Saccharina latissima*. 22nd International Seaweed Symposium. Copenhagen, Denmark.

Safafar, H., Møller, P., Holdt, S. L., & Jacobsen, C. (2015). Microalgal bioremediation of nutrients in wastewater and production of food/feed ingredients. DTU's Sustain Conference. Kongens Lyngby, Denmark.

Safafar, H., Jacobsen, C., & Møller, P. (2014). Antioxidative properties of some phototropic microalgae grown in waste water. 12th Euro Fed Lipid Congress. Montpellier, France.

Safafar, H., Jacobsen, C., & Møller, P. (2015). Lipid profiling of some authotrophic microalgae grown on waste water. 2nd International Conference on Algal Biorefinery. kongens Lyngby, Denmark..

Safafar, H., Jacobsen, C., & Møller, P.(2014). Development of harvesting and up concentration technologies for microalgae as an ingredient in fish feed. DTU Sustain Conference. Kongens Lyngby.

Safafar, H., Jacobsen, C., & Møller, P. (2015). Nutramara conference: Arnessing Marine Bioresources for Innovations in the Food Industry. Dublin, Ireland.

ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ACS	Average cell size
AMB	Ambient
BF3	boron trifluoride
BHT	Buthylated hydroxyl toluene
BOD	Biochemical oxygen demand
CHL	Chlorophyll
CIP	Cleaning in place
C.M	*Chlorella minutissima*
COD	Chemical oxygen demand
C.P	*Chlorella pyrenoidosa*
C.S	*Chlorella sorokiniana*
C.V	*Chlorella vulgaris*
DAD	Diod array detector
De.S	*Desmodesmus sp.*
DHA	Docosahexanoic acid
DM	Dry matter
DPPH	1, 1-diphenyl-2-picryl-hydrazil
DT	Direct trans esterification
Du.S	*Desmodesmus.* sp
DV	Derivatives
DW	Dry weight
EPA	Eicosapentanoic acid
FAME	Fatty acid methyl ester
FFA	Free fatty acids
FLD	Fluorescence detector
FRAP	Ferrous reduction power
FP	Flat panel
GC	Gas chromatography
HPLC	High performance liquid chromatography
HUFA	Highly unsaturated fatty acids
IC	Internal circulation
ICT	Internal circulation tower
ICW	Industrial process water
ISTD	Internal standard
LC-MS	Liquid chromatography-mass spectrophotometry
LC	Long chain
PUFA	Ploy unsaturated fatty acids
MgDVP	Mg-2,4-divinyl pheoporphyrin
N.L	*Nanochloropsis limnetica*
N.S	*Nanochloropsis salina*
PBR	Photo bioreactor
FP PBR	Flat Panel Photo bioreactor
P.T	*Phaeodactylum tricornutum*
PV	Peroxide value
RCF	Relative centrifugal forces
Re	Reynolds number
T PBR	Tubular photo bioreactor
SAFA	Saturated fatty acids
SiC	silicon carbide
Sd	Standard deviation
SM	Spirulina medium
VAC	Vacuum

TABLE OF CONTENT

Chapter 1; Introduction

1.1. Background

Fishmeal is a primary ingredient in the formulation of aquatic feeds, as well as other animal feeds (Thorarinsdottir et al., 2011). Due to the limitations in catches of pelagic fisheries, the continual exploitation of natural fish resource is ultimately unsustainable from both an economic and an environmental point of view (Henry, 2012; Vilegas, 2015). According to Food and Agriculture Organization of the United Nations (FAO), the continuous increase of the world population to over nine billion by 2050, will result in an even higher demand for fish as a vital food resource (Vilegas, 2015). Furthermore, due to the growing awareness of the health benefits of fish in the diet, the global demands for similar healthy food resources and derived products will increase considerably. Fish is a rich source of protein, long-chain fatty acids such as EPA and DHA, iodine and selenium, all of them are not found in plant sources such as fruits and vegetables (Brown et al., 1997; Obach, 2012).

In 2012, more than 16.3 million tonnes of fish (mostly pelagic) were used for fishmeal and fish oil production globally (Henry, 2012). Now it is becoming evident that such exploitation of fish resources will be ultimately unsustainable from both an environmental and economical point of view (Henry, 2012). A continual depletion of this resource, when the global demand for fish and other aquatic product are rising, leads to increasing prices of fish meal and oil. Already now fish meal has developed into an expensive feed (Vilegas, 2015) when compared to other animal feeds, fishmeal being ca. three times higher ($1200/T vs. $300/T for animal feed). In common aquaculture feed now accounts for more than half of the variable operational costs. Even for known commercial plant resources such as corn and soybean, prices raised up over 300% in last seven years (origin oil, 2014). As a consequence, other and more sustainable feed resources with matching essential nutrients such as protein and long chain polyunsaturated fatty acids are critical for the aquaculture industry and in demand. During the past decades, several researchers focused on finding alternative feed ingredients as a replacement of high-cost fishmeal. Hence, the potential competitive feed ingredients must be able to represent the same nutritional profile at a reasonable cost (Henry 2012). Microalgae represent a potential feed source in aquaculture because of their nutritional value closely resembling fish proteins and oil. Microalgae are already the base (first step) of the aquatic food chain and considered as the primary source of bioactive compounds such as carotenoids and fatty acids, which are not being produced by the metabolic system in fish. Algae production requires less space and water (as the water is being recycled) compared to plant resources. The biomass from many algae species has been proven as a rich source of nutrients such as amino acids, essential fatty acids, vitamins and natural antioxidants, offering algae biomass as a promising ingredient in the formulation of fish meal.

1.2.Challenges in utilization of microalgae biomass as a fish feed ingredient

1.2.1. Cultivation

Industrial production of microalgae for the broad application within feed production is still not economical. Among several factors which influence the price of algal biomass, those connected with large-scale cultivation play a major role (Zittelli et al., 2013). The price of growth medium is an important factor which increases the total production costs. Cultivation in open ponds is cheaper than photobioreactors, but the efficiency and biomass productivity are low. Besides that, keeping the quality and purity of the biomass during the cultivation is difficult (Gupta et al. 2015). Closed systems, on the other hand, require a substantial input of energy and automation which increase the overall processing expenses. The overall production cost might be decreased to let the product to be used at reasonable prices. One of the strategies to reduce the overall production cost is to use a low-cost culture medium such as waste water. Some algal species can grow on waste water and produce biomass (He et al. 2013). The waste water used as a growth media for microalgae should be free of pollutants such as heavy metals and pesticides when the resulting microalgae biomass is intended to be used as a feed ingredient. Anaerobic digestion is a modern and promising wastewater treatment technology also for industries. The conversion process happens in an anaerobic sludge reactor with internal circulation (IC). Under anaerobic conditions, methanogenic conversion of organic compounds to biogas will result in methane, CO_2, and an effluent which contains ammonia and many other inorganic compounds. The effluent stream, industrial process water (ICW) depending on the initial wastewater may include a diverse range of compounds (e.g., ammonia). The bio utilisation of the effluent reduces the overall cultivation costs by deduction of the price of growth medium and drinking water. This process also benefits from valorization of the effluent which reduces the environmental polluting load caused by nutrients and thus saves the money which the factory should normally pay for treating the effluent before it can return to the environment.

1.2.2. Choosing the right algae species

It has been estimated that 200,000-800,000 microalgae species in many different genera exist of which about 50,000 species are described. Only very few species have been studied in great detail a suitable source of nutrients for food/feed applications. The screening of microalgae is critically required to select proper microalgae with high biomass productivity and quality. The biochemical composition of the biomass preferably should include high contents of protein (Guedes et al.,2015), long-chain polyunsaturated fatty acids and other required bioactive compounds such as specific amino acids and natural antioxidants, while the contents of carbohydrates should be as low as possible. If waste effluent is being used for cultivation (either as a principal or part of growth medium), the right algae shall be able to efficiently take up the nutrients such as nitrogen and phosphorus and convert them to compounds of interest, e.g., protein.

1.2.3. Downstream processing

Due to the light dependent production, self-shading results in a very low concentration of microalgae in the media, therefore downstream processing of microalgal biomass represents a major cost in the overall production costs. Downstream processing of microalgae is application specific, and there is no globally accepted processing concept of algae biomass for food/feed applications. Thus, an efficient and cheap processing concept is required when the resulting biomass is intended to be used as a fish feed ingredient.

1.2.4. Quality control

With tremendous expansion in the application of microalgae as a source of bioactive compounds, the quality control of microalgae biomass is becoming a significant concern. The quality control criteria and methods for the microalgae during cultivation and industrial applications such as fish feed formulation are necessary to guarantee that the right algae and with the desired biochemical composition are cultivated.

1.2.5. Shelf life and the storage conditions

The storage condition of microalgae biomass is not well studied. Application of microalgae biomass in food, feed or cosmetics requires the knowledge of the optimum and feasible storage conditions to prevent the value-added compounds from deterioration. The understanding of the chemical deterioration under different storage conditions is needed to assist the producers/customers to extend the shelf life of microalgae biomass by choosing correct storage conditions, and thereby value.

1.3 Hypotheses, aim and objectives of the thesis

The overall purpose of this PhD project was to develop a new downstream processing concept for the possible production of biomass from ICW- grown microalgae as a fish feed ingredient. To achieve this goal, the study was further divided into the following sub-studies to cover all required aspects of the project:

i. Developing of rapid fingerprinting strategies for the quality control of microalgae biomass.
ii. Evaluation and characterization of ICW-grown microalgae biomass as a fish feed ingredient.
iii. Cultivation of microalgae on industrial process water as a nutrient source.
iv. Downstream processing of process water grown microalgae for production of a fish feed ingredient.
v. Evaluation of the effects of the storage conditions on the nutritional composition and oxidative stability of dried algal biomass as a fish feed ingredient.

This PhD project tested the following hypotheses (**H1-H4**):

H1; A combination of analytical methods can be developed to be used as a rapid quality control tool for quality control of microalgae biomass.

H2; Some of the microalgae can utilise ICW as the main nutrient source and produce reasonable biomass with high contents of protein, long-chain fatty acids and other bioactive compounds such as natural antioxidants.

H3; A downstream processing concept can be designed for feasible harvest, up-concentration and drying of microalgae biomass with minimum adverse effect on the quantity and quality of its bioactive compounds.

H4; The storage conditions including the storage time, temperature and packaging conditions influence the composition of bioactives and their oxidative degradation.

The following objectives arise from hypotheses;

Screening of microalgae species based on their ability to grow on ICW and to have the desired chemical composition.
Evaluation of growth medium and cultivation duration on the chemical composition of algal biomass to find the proper harvest time and to identify the optimal level of ICW in the growth medium.
To evaluate the effect of large-scale cultivation on the chemical composition and growth of selected species.
To develop and test the strategies for the quality control of the algae biomass during cultivation, processing and storage.
To find the proper storage conditions for microalgae biomass intended to be used as a fish feed ingredient.
To design a specific downstream processing set up and evaluate it in laboratory and large scale.

1.4 Tasks and prerequisites

This PhD study was a part of a big project (FIMAFY) funded by Green Development and Demonstration program (GUDP; project number:34009-13-0616) under the Danish Food Ministry. The GUDP project is a collaboration of various parties including LiqTech A/S, BioMar A/S , EcoLipids ,

IFAU, DTU Aqua and DTU Food. The project aims at developing and testing new technologies to harvest and process microalgae and to demonstrate that the microalgae biomass can be utilized as an alternative resource to fish meal and fish oil for production of fish feed. We established a microalgae cultivation laboratory and designed, constructed and tested pilot scale facilities for the downstream processing trials. Data from laboratory screening trials were implemented to large scale for selected microalgae species. Large scale cultivated biomass was used for the evaluation of storage stability of microalgae biomass. Large scale cultivation was done in kalundborg microalgae facility, in collaboration with Dept. Development, kalundborg utilities and E4W project.

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Chapter 2; Development of rapid fingerprinting strategies for the pre and post-harvest purity control of microalgae biomass

2.1. Introduction

With tremendous expansion in the application of microalgae as a source of bioactive compounds, the quality control of microalgae biomass is becoming a significant concern. Chromatographic fingerprinting has a great potential as a powerful tool for identification, and quality control of microalgae during cultivation and in industrial applications such as fish feed formulation. The performance of single chromatographic fingerprinting seems to be inadequate, e.g. when the unknown species in the culture contributed to the microalgae from the same taxonomic class. Microalgae biomass includes amino acids, fatty acids, pigments, carbohydrates, phenolics, organic acids and vitamins. It has been shown that the amino acid composition of microalgae is not species-dependant (Brown et al., 1997). The composition of phenolics and organic acids are also not completely studied so that could not be used as a clear pattern for the fingerprinting of various species/classes. In this project, a simple and rapid strategy based on fatty acid and pigment compositions is evaluated as a quality control tool for both pre- and post-harvest investigation of microalgal biomass purity.

2.2. Microalgal pigments

During the photosynthesis, the sunlight is absorbed by algal energy-trapping pigments, and transformed into chemical energy. The main pigments, chlorophylls, carotenoids and phycobiliproteins absorb photosynthetically available radiation at the specific range of 400–700 nm wavelengths (Rowan 1989). The pigment composition characterises the different phytoplankton classes (Mangoni et al., 2011). Chlorophylls, carotenoids and phycobiliproteins are present in all photosynthetic algae, but not in most bacteria, protozoa or detritus, allowing phytoplankton to be distinguished from other organisms (Wright and Jeffery, 2006). Some pigments found exclusively in individual classes or genera may serve as useful taxonomic markers (Zapata et al., 2004, Wright and Jeffery, 2006). On the other hand, the particular structure and properties of pigments make their identification challenging. Pigments are sensitive to light, heat, oxygen, and some particular chemicals, so that the environmental, harvesting and processing conditions influence the content and composition of pigments in the microalgae. A variety of pigment isomers or esters could also form in the microalgae cell as a response to growth conditions such as light, nutrients, which makes their identification more complex. The knowledge about the structure, chemical properties and their distribution in various algal classes is a basic requirement for developing a chromatographic method and chromatographic investigation of pigment composition in microalgae.

2.2.1. Chlorophylls and phaeopigments

Chlorophylls have a tetra-pyrroles structure; consist of a large aromatic ring, which contains four pyrrole rings and a magnesium ion (Figure 1). This structure is typically attached to a hydrocarbon tail, excluding chlorophyll c (Mulders et al., 2014). There are several different types of chlorophylls (a, b, c, d, and f). In each type of chlorophyll, the molecules attached to the tetrapyrroles structure (chlorin) are different. These small differences lead to differences in the absorption spectrum and therefore visual realization, chlorophyll-*a* appears blue-green, chlorophyll-*b* brilliant green, chlorophyll-*c* yellowish green, chlorophyll-*d* bright/forest green, and chlorophyll-f emerald green (Chen et al., 2010, Mulders et al., 2014). When the unstable central magnesium is removed (due to oxidation, heat treatment, etc.), the colour of the new structure (phaeophytin) turns to pale brownish-dusky (Humphrey, 2004), which subsequently reacts with metals such as copper to form a more stable blue-green complex, known as copper-phaeophytin (Humphrey, 2004). The structure of chlorophyll-*a* and phaeophytin-a are showed in **Figure 2.1.** In general, various products form from de-gradation of chlorophylls. Thermal degradation is more insidious and occurs progressively at temperatures above 60 °C to give other forms of degradation products such as pyrophaeophytin. When the phytyl group (which is a C20 di-terpene) is removed by enzymes (chlorophyllase), or acid-base hydrolysis, chlorophyllide is formed. Hydrolysis of phaeophytin results in the formation of phaeophorbide (Humphrey, 2004). Liquid chromatography is the preferred method for the identification and detection of chlorophylls and their derivatives. Due to the various color absorbance spectra, degradation products of chlorophylls could easily interfere in the spectrophotometric analysis of carotenoids. Thus, the spectrophotometric methods could not provide reliable-realistic results, particularly in heat treatment trials or in storage experiments, when the oxidation occurs in the biomass. On the other hand, changes in the chemical structure shift the polarity of the molecule, which makes the extraction and analysis more accurate and complex. The extraction and analysis of the chlorophylls shall be fast, and without application of heat, to prevent the degradation of the chlorophylls as described above.

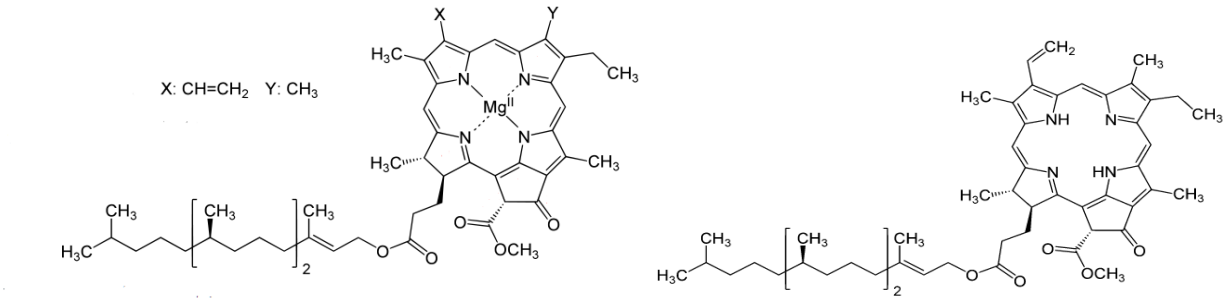

Figure 2.1. Structure of (a) Chlorophyll-*a*, and (b) Phaeophytine-*a*

2.2.2. Chlorophylls diversity in microalgae

Chlorophyll-a, which is the primary light harvesting pigment, is found in all photosynthetic organisms, including algae (Mulders et al., 2014). In contrast, chlorophyll-b is particularly discovered in the Chlorophyte and their descendants (Mulders et al., 2014). Chlorophyll-*c* (either as *c1, c2 or c3*) could exclusively be found in the descendants of the Rhodophyta (Jeffrey and Wright 2005). Polypeptides of chlorophyll-*a* or -*c* are present in the algae from divisions such as Heterokonta, Haptophyta, Bacillariophyta, Phaeophyta, Cryptophyta and Dinophyta (Zapata et al., 2006). Some of the chlorophyll related compounds such as [DV]-P Chlide-*a* (Mg-2,4-divinyl pheoporphyrin) is reported from green prasinophytes (Zapata et al., 2006). Peridinin is a carotenoid complexed with chlorophyll-a, and found exclusively in peridinin-containing Dinophyta (Mulders et al., 2014). Thus the chlorophyll profile of the microalgae gives remarkable data about the sample when the analysis is done properly. Chlorophyll-*c* and MgDVP are highly polar compounds, which occur early in the chromatogram and very close to each other. Therefore, the chromatographic separation, identification and quantification of the isomers require well-developed methods.

2.2.3. Phycobiliproteins

The phycobilins most commonly found in microalgae are phycocyanobilin and phycoerythrobilin. Phycocyanobilin is the major component of the phycobiliproteins phycocyanin (deep blue) and allophycocyanin (light blue), whereas orange-red phycoerythrobilin is the major component of phycoerythrin (Mulders et al., 2014). The main difference between the compounds is associated with the compounds bound to the pyrrole rings, which cause differences in color. Phycobiliproteins are usually found in pigment aggregates called phycobilisomes, which contain hundreds of poly peptides.

2.2.4. Carotenoids and their isomers

Carotenoids are a class of terpenoid pigments and appear yellow to red in color. Their main structure consists of a 40-carbon polyene chain as the molecular back- bone. This structure is associated with carotenoid's chemical properties including light-absorption features that are essential for photosynthesis and the stability of the molecule (Guedes et al., 2011). This structure may include cyclic groups (rings) and/or oxygen-containing functional groups. Two core groups of carotenoids are carotenes, which are true hydrocarbons, and xanthophylls, which additionally contain oxygen atoms groups (**Figure 2.2**). In xanthophylls the oxygen is present in various forms such as –OH groups (e.g., lutein); oxi-groups (e.g., canthaxanthin) or both (e.g., astaxanthin) (Guedes et al., 2011). All of the xanthophylls, which are synthesized by plants such as violaxanthin, antheraxanthin, zeaxanthin, neoxanthin and lutein, can also be synthesised by microalgae. However, individual xanthophylls such as loroxanthin, diatoxanthin, diadinoxanthin and fucoxanthin can only be found in algae or diatoms

(Eonseon et al., 2003). The carotenoids, which are involved in photosynthesis, such as α-carotene, peridinin and fucoxanthin, are known as primary carotenoids, while others, including β-carotene, lutein, zeaxanthin and diadinoxanthin play mainly a photoprotective role (Dawson, 2007). Primary carotenoids in most of the green algae are synthesised within plastids, and accumulate there, while secondary xanthophylls in some green microalgae (for example astaxanthin in *Haematococcus* sp.) accumulate in the cytoplasm.

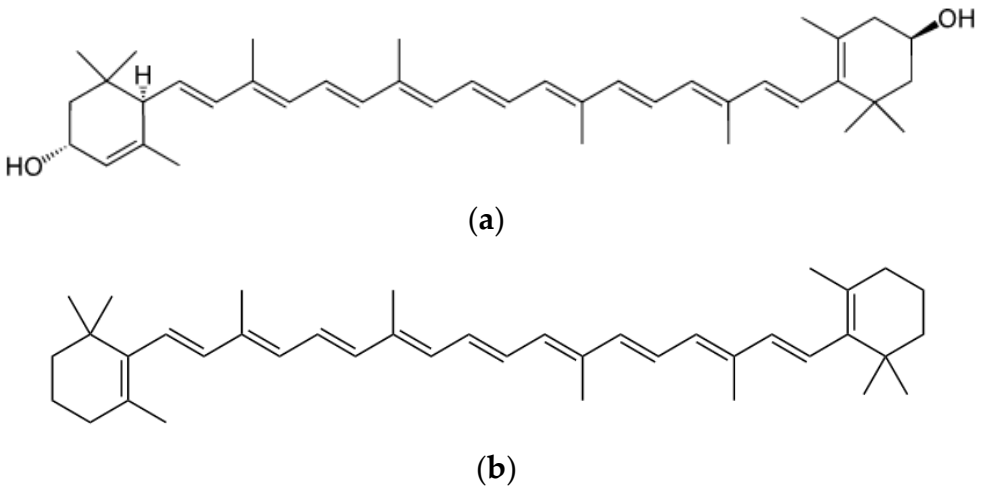

Figure 2.2. The structure of (**a**) Lutein (3R,3′R,6′R-β,ε-carotene-3,3′-diol), and (**b**) α-carotene; β-carotene (*beta*,*beta*-Carotene).

Alternatively, xanthophylls which are synthesised in the chloroplast may be transferred and build up in the cytoplasm (Guedes et al., 2011). Thus, they may be found in all cellular compartments. Xanthophylls are semi-hydrophobic compounds typically associated with thylakoid membrane, bound to specific proteins or localised in lipid vesicles (plastid stroma or cytosol (Guedes et al., 2011). Carotenoids perform several functions in microalgae: they are associated with photosynthesis and contribute to the function of photosynthetic complexes by quenching the excited chlorophylls (triplet states), scavenging reactive oxygen species and dissipating excess energy (paper 1). However, carotenoids are very sensitive and easily decompose, during the extraction, so that the extraction method should be capable of extracting all of the carotenoids distributed within cell components, without significant loss.

2.2.5. Esterified xanthophylls

The esterification of individual xanthophylls (not carotenes) takes place in some microalgae and plant cells. A xanthophyll ester (lutein di-palmitate) was first identified by Kuhn and Winterstein in 1930 (Pérez-Gálvez and Mínguez-Mosquera, 2005). The pathways and mechanisms of this esterification process are not known, although it is assumed that xanthophylls become esterified by

acyl–coenzyme A. By esterification, the microalgae metabolic system increases the liposolubility of (mostly secondary) xanthophylls. It has been reported that as enzymes involved in carotenoid biosynthesis are membrane-bound a higher liposolubility facilitates biogenesis of carotenoids from their parent compounds. It is remarkable that xanthophylls are preferentially esterified by particular fatty acids, such as C20:0 and C18:1, whereas others (C18:3) do not participate in the esterification process (Pérez-Gálvez and Mínguez-Mosquera, 2005). It could be hypothesized that by this, the lipids could be protected against the oxidation, by the antioxidative effects of these xanthophyll pigments. The esterified astaxanthins, vaucheriaxanthin and lutein are reported in various species such as *Haematococcus* sp., *Nannochlorosis* sp. and *Chlorella* sp. (Del Campo et al., 2004; Mulders et al., 2014). The esterification can happen once or twice, providing a set of compounds with different polarity and molecular weights. Although esterification does not change the chromophore properties of the carotenoid molecule, it does modify the immediate molecular environment, increasing the liposolubility, so that chemical activities may also alter, depending on the type and number of fatty acids bound to the xanthophyll. Hence, their retention time would be different during chromatographic separation, and could easily overlap with other compounds with the same retention time.

2.2.6. Carotenoids diversity in microalgae

The presence or absence of particular carotenoids has been used for class or order distinctions within some chlorophytes (Fawley and Lee 1990; Guillard et al., 1991). It has been shown that the distributions of some carotenoids (loroxanthin, siphonaxanthin, siphonein) are disjunct and do not have an apparent systematic utility (Fawley and Lee, 1990). β-carotene which is one of the most known carotenoids, could be found in most cyanobacteria and microalgae excluding the Cryptophyta and bilin-containing Dinophyta (**Table 2.1**). But the capability of the production of β-carotene in high concentrations is limited to a few species such as *Dunaliella* sp. from Chlorophyta (Mulders et al., 2014). Astaxanthin is an exclusive xantophyll of the Chlorophyta, and could be produced in high concentrations by some species such as *Haematococcus* sp. and *Chlorella zofingiensis* (Liu et al., 2014). Lutein is another xanthophyll which exclusively is found in Chlorophyta and few green Dinophyta. In some of the Chlorophyta and under certain conditions lutein can be converted to loroxanthin (Jeffrey and Wright, 2005). Lutein can be produced in extremly high concentrations by *Chlorella vulgaris* (paper 4). Fucoxanthin is found in the Haptophyta, some Dinophyta, the Chrysophyta, and the Bacillariophyta(Mulders et al., 2014). In addition, dinoxanthin is also found in these Dinophyta. Zeaxanthin is found in low concentrations in Prochlorophytes, Chlorophytes, Prasinophytes, Euglenophytes, Chrysophytes, Raphidophytes, Cryptophytes and eustigmatophytes and even Chlorophytes (Millie et al., 1994; paper 1). This pigment is a marker for cyanobacteria such as *Arthruspora* sp. Vaucheriaxanthin and their esters are exclusively found in eustigmatophytes and rarely in some chrysophytes (Milie et al., 1993; Wright and Jeffrey, 2006; papers 1 and 3). Fucoxanthin

and its derivatives are shown as good markers of Diatoms (Wright and Jeffrey, 2006). However fucoxanthins also present in low concentrations in some haptophytes, chrysophytes, raphidophytes, bolidophytes and dinoflagellates (Mulders et al., 2014).

Table 2.1. Pigment diversity in different microalgal phyla (Mulders et al., 2014; Millie at al., 1993; Wright and Jeffrey, 2006). (• = present).

Phylum	P-cy	P-er	A-cy	Lu	a-C	b-C	Ze	Ly	An	Vi	Ne	Ca	As	Dt	Di	Dn	Pe	Fu
Chlorophyta				•	•	•	•	•	•	•	•	•	•					
Euglenophyta						•					•			•	•			
Green dinophyta				•		•	•			•	•							
Cyanophyta	•	•	•			•	•	•				•						
Chrysophyta						•	•		•	•				•	•			•
Glaucophyta	•	•	•			•	•											
Cryptophyta	•	•																
Rhodophyta	•	•	•			•	•											
Bacillariophyta						•								•	•			•
Haptophyta						•								•	•			•
Billin dinophyta	•	•																
Peridinin dinophyta						•								•	•	•	•	
Fucoxanthin dinophyta					•	•									•			•
Xanthophyta						•				•	•				•	•		

P-cy= phyco cyanidine; P-er= phyco erythrine, A-cy= allophycocyanin; Lu = Lutein; a-C= α-Carotene; b-C= β-Carotene; Ze= Zeaxanthin; Ly= Lycopene; An= Antheraxanthin; Vi= Violaxanthin; Ne= Neoxanthin; Ca= Canthaxanthin; As= Astaxanthin; Dt= Diatoxanthin; Di= Dinoxanthin; Dn= Diadinoxanthin;Pe= Peridinine; Fu= Fucoxanthins.

Finally, loroxanthin as a rare xanthophyll is an occasional component of chlorophytes and a good marker for chlorophytes in Antarctic area (Wright and Jeffrey, 2006).

Based on the above, the identification and characterization of microalgae classes based on the pigment composition is a reliable approach, compared to the microscopic detection of indicator algae (Wright and Jeffrey, 2006). Chlorophylls, carotenoids, and phyco-pigments (preferably as a combination) have been widely used for chemosystematics identification of phytoplankton assemblages (Millie et al., 1993). Identification of algal phylogenetic groups through pigment signatures is mostly limited to the division or class levels. For a known species and regardless of environmental variations, monitoring of the algal pigment composition could be an important quality control measure. A practical example of the detection of contamination by unwanted microalgae during the large-scale cultivation is shown in **Figure 2.3,** where a typical pigment composition of a pure culture (day 10) of *Nannochloropsis salina*

(Eustigmatophyte), is compared with the same culture after contamination (day 18). The samples were grown in 4000 L flat panel photobioreactor as described in paper 3. The presence and almost similar concentration of lutein, and chlorophyll-b indicated the presence of a Chlorophyte such as *Chlorella* sp. On the other hand, the contents of violaxanthin, vaucheriaxanthin (both free and esterified), which are typically present in Eustigmatophyte, decreased compared to day 10. The contamination could be very severe, leading to a biomass with completely different biochemical composition.

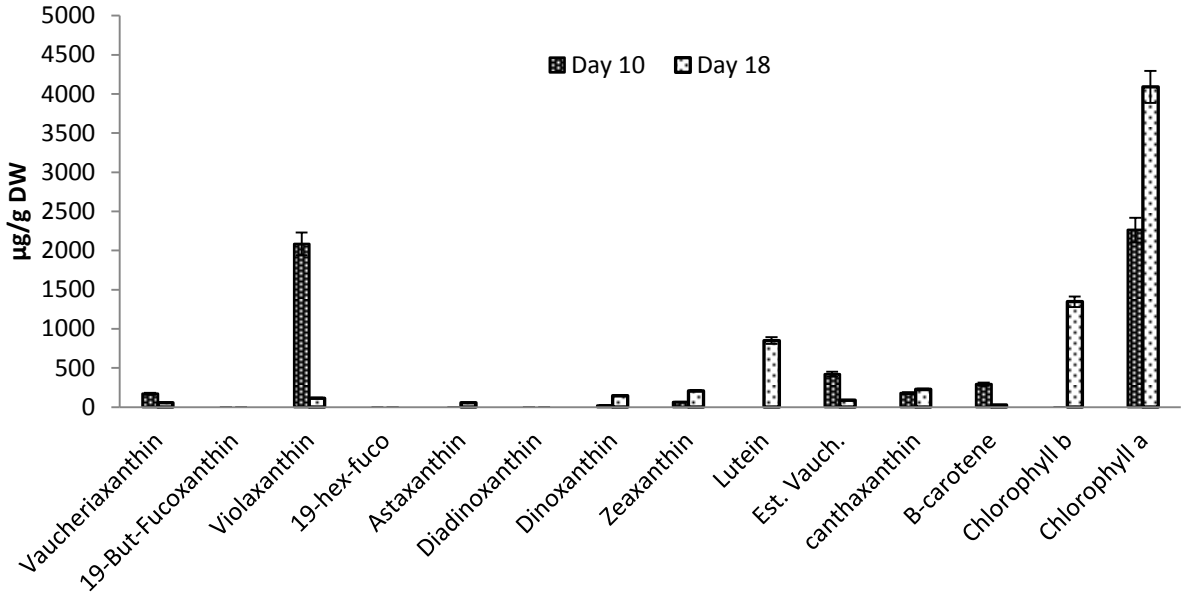

Figure 2.3. An example of the contamination during the large scale cultivation of *Nannochloropsis sp.* Results are expressed in µg pigment concentration per g of sample in dry weight. Error bars represents the standard deviations. Data from the large scale cultivation experiment in Kalundborg microalgae facility (Kalundborg, Denmark). See paper 3 for more details of growth conditions. This experiment was stopped, discarded and repeated because of the contamination.

2.2.7. HPLC analysis of microalgal pigments

Due to the fact that some pigments span many algal classes, interpretation of pigment data is difficult, so specific techniques should also be used to improve the extraction and identification. It has been demonstrated that liquid chromatographic pigment analysis is a powerful and reliable technique for nano- pico sized phytoplankton for which the identification is not possible by microscopic evaluation (Wright and Jeffery, 2006; Claustre et al., 2006). Several HPLC procedures have been developed to date, each having its pros and cons. Nineteen HPLC methods were published between 1983- 1998 as reported by Jeffrey et al. (1999), followed by methods developed by Zapata et al. (2000) and Van Heukelem and Thomas (2001). No single HPLC method is capable of fulfilling the requirements for all applications (Bidigare et al., 2005). Many factors affect the sensitivity, accuracy,

trueness and repeatability of a method. The particular method is required to fulfil the requirements of a specific application. In addition to the chromatographic separation, identification and quantification, the method should also include an integrated well-designed extraction procedure to guarantee the trueness of the results. The extraction shall be fast efficient and able to recover all intended target pigments without significant loss. The extraction solvent(s), on the other hand, shall be compatible with the HPLC conditions (e.g. the solvent mixture). The modification or development of a proper procedure requires the following information; target compounds/ purpose of the analysis, intended microalgal species (if specific species are being analysed), precision/trueness criteria, required analysis time, sample conditions (size, moisture content, etc.), as well as any specific in-house possibilities and limitations. Details of the extraction and liquid chromatographic analysis methods which were used in this project are shown in paper 1. The chromatographic separation method was a mofication from Van Heukelem and Thomas (2001). The main features of the modified method include the extraction method and the internal standard which was used for volume correction.

2.2.8. Extraction method

Acetone and methanol are the main solvents used for the extraction of pigments. Methanol has some advantages for extraction, such as lower volatility compared to acetone, and also produces peaks with higher resolution compared to the pigments extracted in acetone (Wright and Jeffrey, 2006). Methanol is also superior for extracting Chl *b* and chlorophyll degradation products. No degradation was observed in the pigments extracted by methanol containing BHT within 20 hours residence time at cold autosampler tray (4°C). It was shown that methanol can disintegrate cell membranes more than other solvents (**Figure 2.4**).

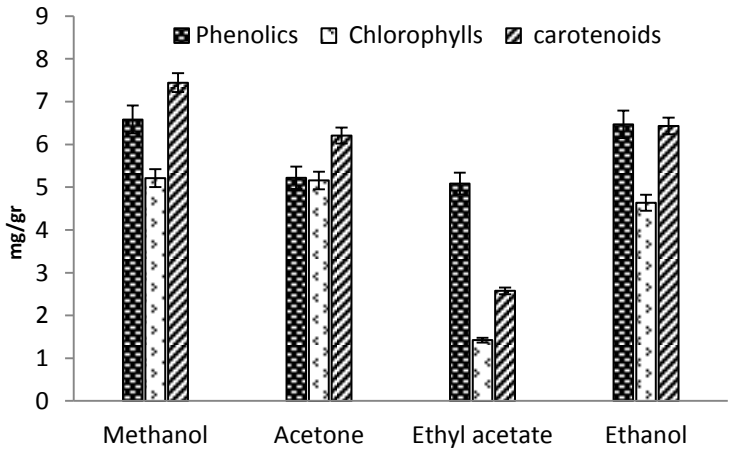

Figure 2.4. The extraction efficiencies of different solvents for phenolic compounds and pigments. Samples of *Chlorella sorokiniana* were used for the study (paper 1 for details)

In the present study of pigments, development of chlorophyll derivatives (e.g., phaeopigment) was not detected due to the low extraction time, which was achieved by the assistance of microwave power. For details see paper 1.

2.2.9. Internal standard

Butylated hydroxytoluene (BHT) was used as internal standard (ISTD) for volume correction. BHT is also a powerful antioxidant so the degradation of the carotenoids was limited during the extraction and analysis. The absorbance of BHT was monitored at 280 nm; it has no absorbances at 450 nm and 670 nm where the carotenoids and chlorophylls are being detected, respectively(**Figure 2.5).** Therefore, no interferences happened at wavelengths used to quantify pigments. On the other hand, BHT is completely dissolvable in methanol, which was used as the main solvent, providing very nice and sharp peaks when compared to the tocopherol acetate which is typically used as ISTD. Calibration was performed using individual pigment standards, which concentrations have been certified by the reference laboratory of DHI A/S (Hørsholm, Denmark).

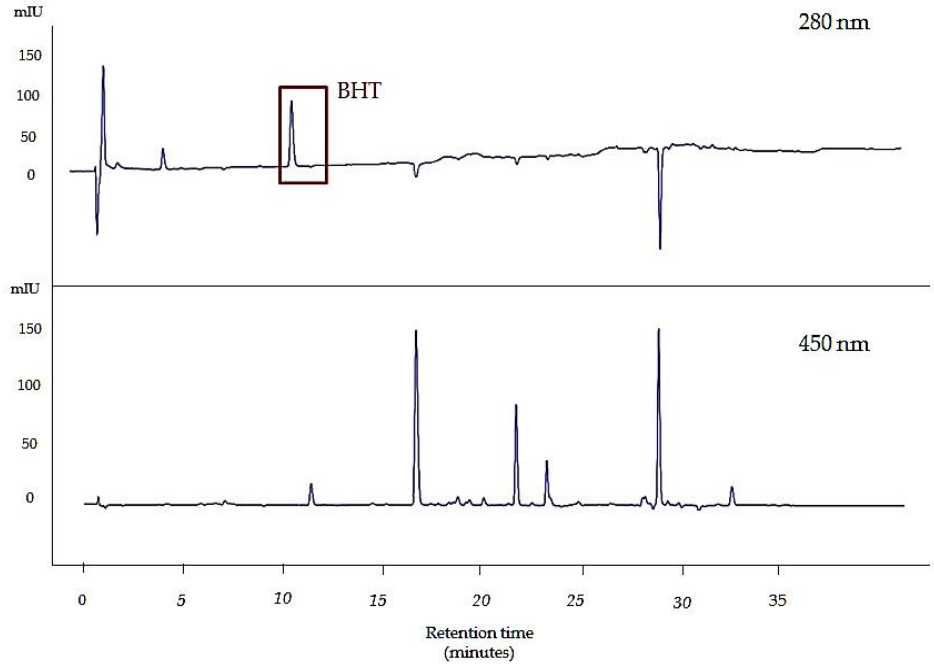

Figure 2.5. The retention times of internal standard (BHT) and pigment peaks. The separation performed on a sample of *Nannochloropsis limnetica,* obtained during laboratory cultivation experiments used in paper 1.

The DHI standard mixture was also used for the evaluation and optimization of the chromatographic separation (**Figure 2.6**). DHI pigment standard mixes include most of the chlorophylls (and pheophytine) and carotenoids. The pigment standard mixes play a major role in the improvement of the methods which are extensively being used for the pigment analysis in microalga.

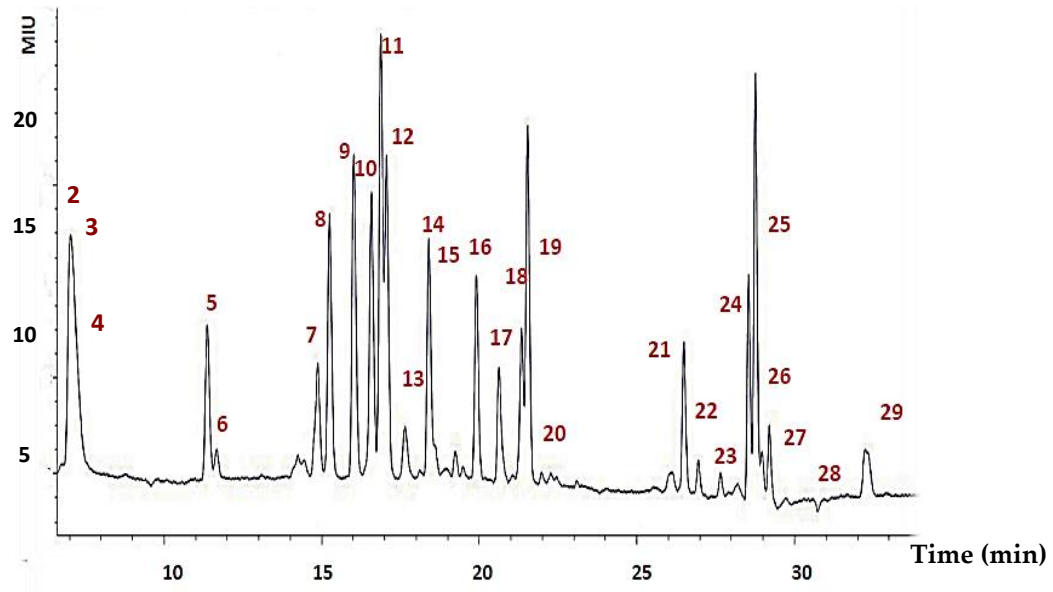

Figure 2.6. DHI pigment standard mix 1, separation and identification by the modified HPLC method (paper 1). Separation was done by the conditions described in paper 1. **Pigment peaks:1-** chlorophyll-*C3*(is not shown here)**, 2 to 4-** chlorophyll-*C2*, Mg DVP and chlorophyllide-*a*, respectively **5-** peridinin, **6-** peridinin isomer, **7-** 19-but-fucoxanthin, **8-** fucoxanthin, **9-** neoxanthin, **10-** prasinoxanthin, **11-** violaxanthin, **12-** 19-hex-fucoxanthin, **13-**astaxanthin, **14-** diadinoxanthin, **15**- dinoxanthin, **16-** alloxanthin, **17-** diatoxanthin, **18-** zeaxanthin, **19-** lutein, **20-** di-hydrolutein, **21-** DV-CHL-*b*, **22-**CHL-*b*, **23-**crocoxanthin, **24-**DV CHL-*a*, **25**- CHL-*a*, **26-** DV CHL-*a* epimer, **27-** CHL-*a* epimer, **28-** phaeophytin-*a* , **29-** β-carotene.

2.3. Fatty acid composition data as taxonomic marker of microalgae

The fatty acid composition of microalgae has been proven as a potential tool for the identification of macroalgal taxa (Mourente et al., 1990). Among the various biochemical markers, the fatty acid composition is considered as a marker to define groups of different taxonomic ranks in flowering plants, Embryophytes and recently microalgae (Lang et al., 2011). Viso et al. (1993), evaluated fatty-

-acid compositions of 28 marine microalgae from nine different taxonomic classes. The study demonstrated that a combination of several criteria (C16, C18, C20 and C22 fatty acids and their proportions) must be used to discriminate taxonomically categories of microalgae, based on fatty acid compositions. Some other publications have also demonstrated that cultured microalgae show different fatty acid compositions depending on their taxonomic position (Schweder et al., 2005; Rossi et al., 2006). In chemotaxonomic investigations using fatty acids, the traditional approach has been to seek for one or more specific fatty acids and use either these or their proportions or even both as indicators for identification (Mongrand et al., 2005). However, the fatty acid composition may not be reliable enough when used as a single marker to distinguish among different genera and species (Lang et al., 2011). It has been shown that both environmental and growth medium influence the variations of fatty acids during the cultivation (papers 3 and 4). The evaluation of pigments and fatty acids as a combined tool provides more reliable results for the investigation of the purity of microalgae biomass. As a practical example, **Figure 2.7** represents the typical fatty acid compositions of pure (day 10) and contaminated (day 18), samples of *Nannochloropsis salina*, which was previously described (**Figure 2.3**). Again a typical fatty acid composition of an eustigmatophyte which mostly includes C16 and C20 fatty acids with high contents of C20:5 represent a pure culture at day 10.

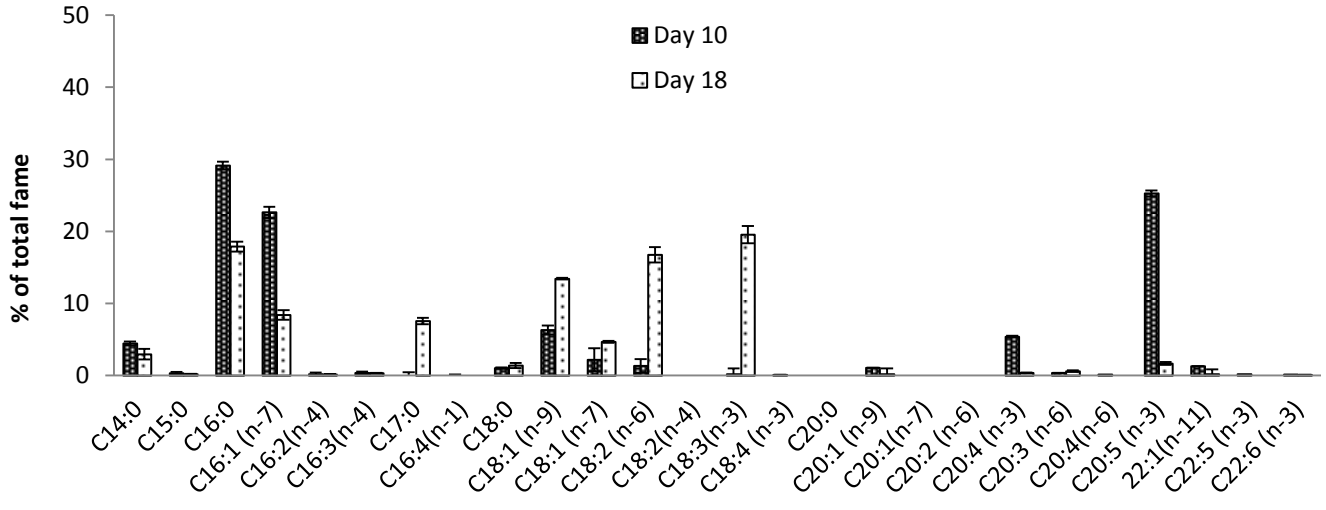

Figure 2.7. Fatty acid compositions (% of total fatty acids) of pure and contaminated samples of *Nannochloropsis salina*. Data from the large scale cultivation experiment in Kalundborg microalgae facility (Kalundborg, Denmark). See the paper 3 for more details of growth conditions.

However, in the sample from day 18 high proportions of C18 fatty acids as well as the low concentration of C20:0 fatty acid(particularily C22:5 n-3) suggested that a chlorophyte, such as *Chlorella* sp. had overgrown the culture. In general, fatty acid composition can be used as

chemotaxonomic markers, albeit not at the levels lower than classes (**Figure 2.8**). Fatty acid composition is also an useful tool for the study of growth stages, or conditions (**Figure 2.9**), and also to investigate the effect of storage time and conditions (papers 3-5).

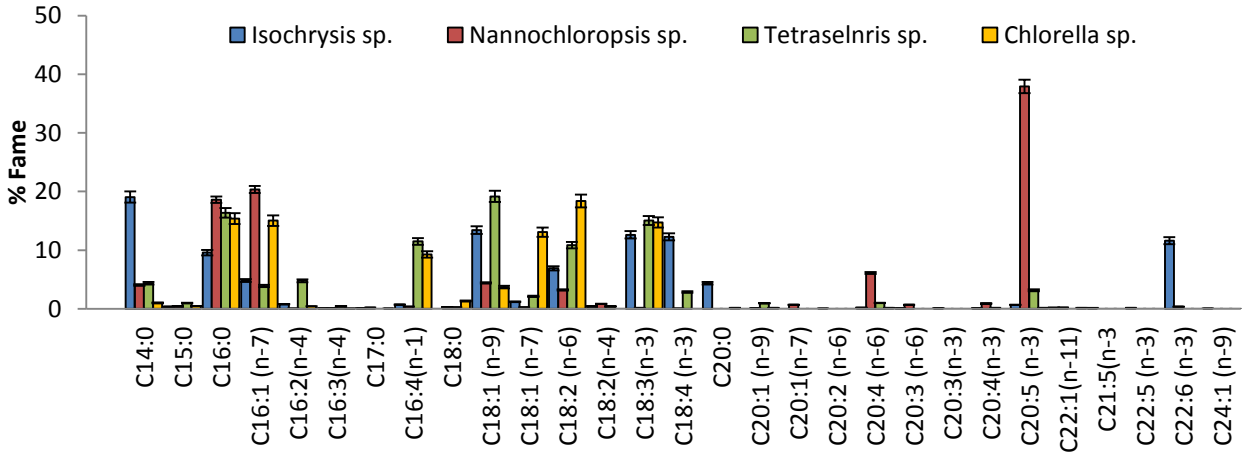

Figure 2.8. Fatty acid composition of four microalgae biomass from different classes, *Nannochloropsis* sp. (Eustigmatophyceae); *Isochrysis* sp. (Prymnesiophyceae); *Chlorella* sp. (Trebouxiophyceae); and *Tetraselmis* sp. (Prasinophyceae). Error bars show the standard deviations (n=4). Data presented as normalized % of each individual fatty acid per total fatty acids.

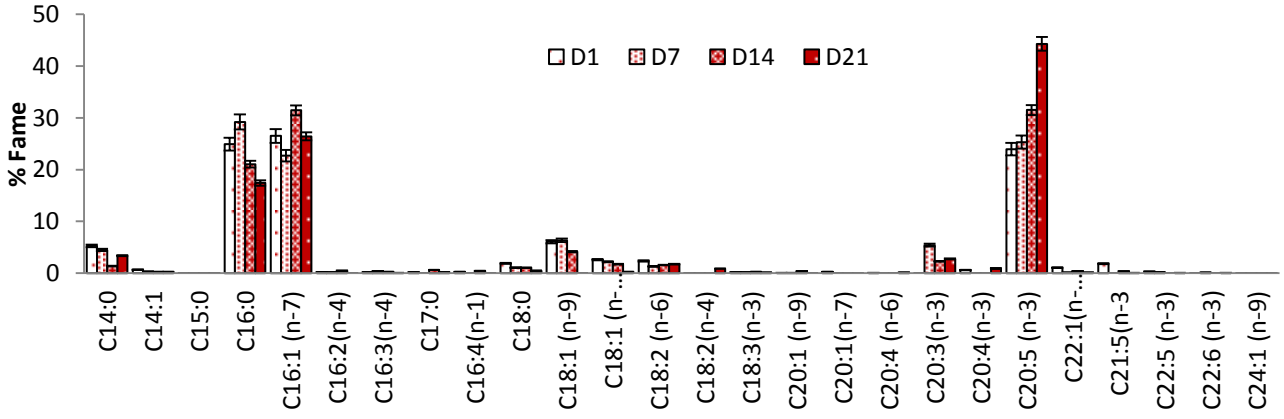

Figure 2.9. Fatty acid composition of large scale cultivated *Nannochloropsis* sp. (Eustigmatophyceae); during the growth (n=4). Samples were taken at the first day of cultivation (D1) to the 21st day of cultivation (D 21). Data are presented as normalized % of each individual fatty acid per total fatty acids. For more details see paper 3.

Application of fatty acid composition as a fingerprinting tool requires fast, reliable and precise methods. Standard methods methods require solvent extraction of lipids and subsequent transesterification of the fatty acid in extracted lipid fraction. The procedure is very long and requires high volumes of solvents and samples. In contrast, direct trans esterification (DT) methods are fast and require low amounts of sample that could be used for the detection of contamination, or for the investigation of deteriorations during the applications and storage. The DT method developed in this project (paper 2) omitted lengthy lipid extraction. Fatty acid methyl esters (FAME) were prepared directly from base-catalyzed methanolysis followed by BF3 methylation reactions. The GC analysis of DT-FAME revealed fatty acid composition virtually identical to that of FAME by the standard method (**Figure 2.10**).

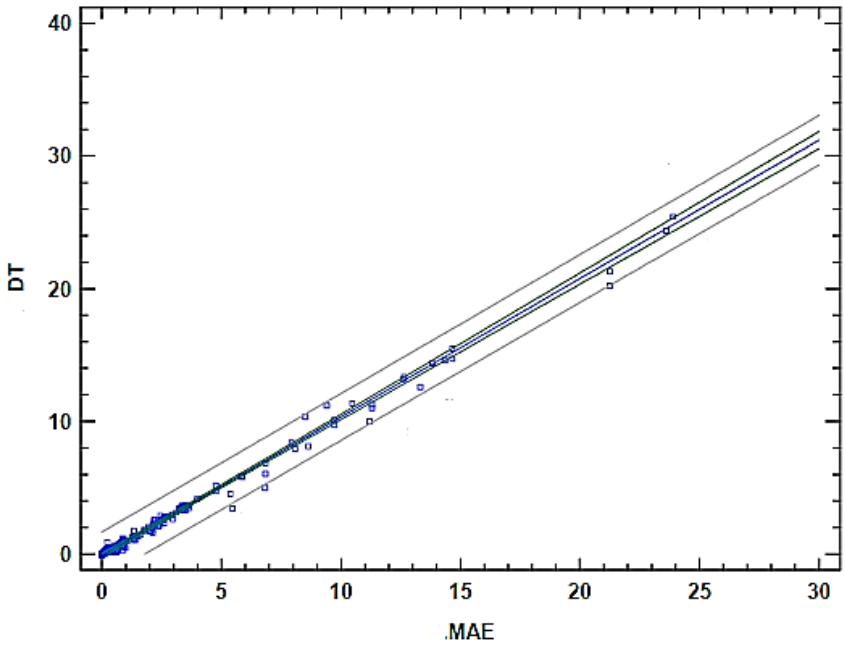

Figure 2.10. Correlation of the fatty acids composition of various microalgae species (n=10), between direct transesterification method and the microwave assisted reference standard method(R-squared = 99.0 %, α=0.05%, and n=10). See paper 2 for details.

2.4. Conclusion

Liquid chromatographic analysis of the pigments is currently one of the best means to evaluate of the purity of microalgae. Pigment analyses have proven to be an effective means for rapidly characterising algal groups in both pre and post-harvest applications. The DT method which is presented in this project could be suggested as a rapid and routine control measure to verify the

quality and/or purity of microalgae biomass during cultivation or application as a food/feed ingredient. The combination of fatty acid and pigment composition provides very useful data, which might be used as a tool for the investigation of the purity or deterioration of the microalge biomass.

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Chapter 3; Selection of microalgae biomass as a fish feed ingredient

3.1. Introduction:

Algae are known as the base of the aquatic food chains providing the food resources for aquatic animals such as fish. Due to the individual chemical composition, microalgae have been indicated as likely sources of protein, lipids and bioactive compounds such as carotenoids (Spolaore et al., 2014; Becker, 2007). Algal protein and lipid contents ranged between 20-70 %DW, and 5-40 %DW, respectively. The variations in algal chemical composition highly depend on parameters such as species, growth condition and culture age (Brown et al., 1997). Algae are an extremely diverse group of organisms; thus it is necessary to investigate the particular qualities of specific algae.

This study aimed at screening and evaluating various microalgae species grown on waste water for their chemical composition based on protein (amino acid composition), lipids (fatty acid composition) and other bioactive compounds, respectively and in order or importance. The aim was to select the species most suitable for utilization as a fish feed ingredient. The target biomass may be obtained from one or more species and include high contents of protein and moderate contents of lipids containing long-chain polyunsaturated fatty acids (LC PUFA). The content of natural antioxidants such as carotenoids, tocopherols and phenolic compounds is also considered an important factor for the selection of proper species due to the protective function of these compounds against oxidation-related damages of the LC PUFA. This is an initial element in the formulation of aquatic feeds. (Papers 1 and 6).

3.2. Biomass samples

Samples of the waste grown algal biomass were achieved from various cultivation trials as below;

Chlorella sorokiniana ; C.S, (CCAP 211/8K) as in papers 1.
Phaeodactylum tricornutum; P.T, *Dunaliella salina;* Du.S, *Nannochloropsis limnetica;* N.L, *Desmodesmus* sp.; De.S as in paper 1.
Nanochloropsis salina; N.S, (SAG: 40.85), as in paper 1 and 3.
*Chlorella pyrenoidosa; C.*P (ATCC®75668™) and *Chlorella vulgaris* (SAG 211-81), as in paper 4.
Chlorella minutissima; C.V (NIVA-CHL 183) as described in chapter 4 (unpublished data).

Cultivation trials were done at the laboratory (0.5- 5 L), and large scale (*for Nannochloropsis salina* and *Chlorella pyrenoidosa*), (see papers 1, 3 - 4 and Chapter 4 for more details).

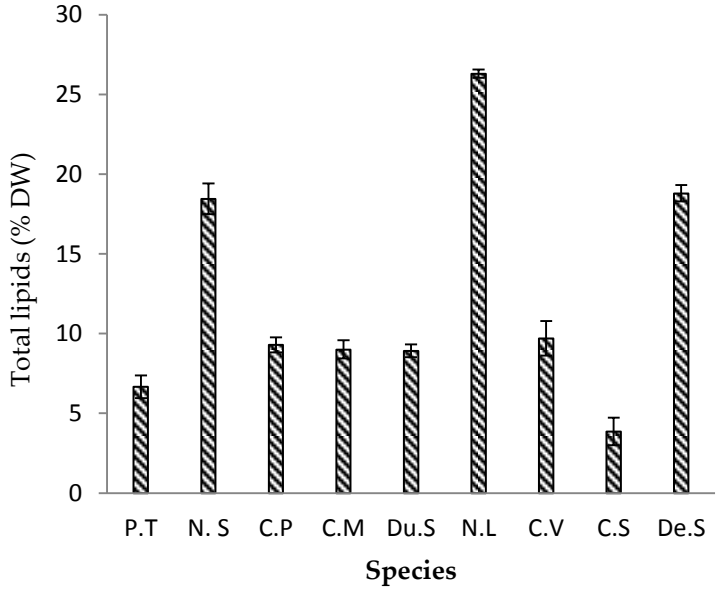
3.2. Analytical methods

All methods are described in papers 1 and 2. Furthermore, the methods implemented for pigment and fatty acid composition analysis are described in details in chapter 2.

3.3. Results and discussion

3.3.1. Lipids

Variations in the contents of total lipids were highly influenced by the species, cultivation duration (so-called culture age), and growth conditions and growth medium composition (papers 3-4). In nitrogen-rich media, less lipid (and carbohydrates) is being produced by the microalgal cell. Phosphorus is another essential nutrient in growth media, which promotes the growth of microalgal cell and enhances the lipid formation. In general, it has been shown in numerous studies that higher lipid contents are achievable in nitrogen-starved cultures (Hu, 2004). It has been reported that under nitrogen starvation condition, cell metabolism shifts from protein synthesis to either lipid or carbohydrate synthesis pathways (Hu, 2004). From a taxonomic point of view, under nitrogen limitations, some species accumulate carbohydrate rather than lipids. As an example, *Dunaliella* sp. produces large quantities of mono-, di- and polysaccharides and glycerol under nitrogen deprived growth conditions (Borowitzka & Borowitzka, 1988). For a single genus of Chlorella, some strains were found to accumulate high contents of carbohydrates, under nitrogen starvation, whereas others accumulated neutral lipids instead (Richmond, 1986). Carbohydrate is not a target compound in the algae biomass as a fish feed ingredient, so it is crucial to avoid species which accumulate carbohydrates. Typical lipid contents of the species tested in this PhD project are shown in **Figure 3.1**. Lipid content values corresponded to the samples from the nearly same growth stage (early stationary growth phase). It can be seen that green algae (Chlorophyceae) excluding *Desmodesmus* sp., and also the diatom *Phaeodactylum tricornutum* accumulates lower contents of lipids, compared to eustigmatophytes such as *Nannochloropsis salina* and *Nannochloropsis limnetica*. Biochemical studies have shown that acetyl-CoA carboxylase (ACCase) catalyses an early step in fatty acid biosynthesis, may be involved in lipid accumulation process (Roessler et al., 1994). The low lipid contents at the early stationary phase, when the nutrient resources have declined, might be associated with accumulation of carbohydrates in a microalgal cell which is not favoured when the biomass is intended to be used as a fish meal ingredient. It has been demonstrated that microalgae from the class Scenedesmaceae (such as *Desmodesmus* sp.*)* and the class eustigmatophyte (*Nannochloropsis* sp.*)* accumulate more lipids compared to green algae such as *Chlorella* sp. (Becker, 1994). The high lipid content can be a benefit when it includes LC PUFA such as C22:6 n-3 and C20:5 n-3. In this project, microalgae *Nannochloropsis salina* representing a biomass with high content of C20:5 n-3 was selected as source of LC PUFA.



3.1. Total lipid contents in waste-grown microalgae species. Samples of microalgae were harvested at the same growth stages (early stationary). Error bars show the standard deviations, n=2. *Chlorella sorokiniana* (C.S); *Phaeodactylum tricornutum (*P.T*); Dunaliella salina (*Du.S*); Nannochloropsis limnetica*(N.L)*; Desmodesmus* sp. (De.S)*; Nanochloropsis salina (N.S); Chlorella pyrenoidosa (C.P); Chlorella vulgaris* (C.V);*Chlorella minutissima* (C.M).

3.3.2. Protein content

In a growth media with sufficient available nitrogen, carbon fixated in the photosynthesis process is being used for protein synthesis (papers 3-4). Thus, the growth media directly influences synthesis of nitrogen-containing compounds such as protein, nucleic acids, amino acids, and chlorophyll depending on its level of nitrogen. This positive correlation between the protein content and concentration of nitrogen was already reported by various studies (Hu, 2003; Lv et al., 2010; He et al., 2013; Guccione et al., 2014). Accumulation of carbohydrates or lipids will start when the nitrogen declines beyond a threshold, which is species-specific (Griffiths et al., 2014). Similar to the lipid content, the protein content in the microalgae is also species-dependant (Hu, 2004). In **Figure 3.2** protein content in various microalgae species is shown. The simple comparison of the protein contents represents the *Chlorella* sp. as the species with the highest protein accumulation, excluding the *Chlorella sorokiniana*. The highest protein content was observed in *Chlorella pyrenoidosa*, followed by *Chlorella vulgaris* and *Chlorella minutissima*, respectively. Such high protein content in *Chlorella* sp. was previously reported (Becker, 2007). Both lipid and protein contents in *Chlorella sorokiniana* are low, which suggests that these algae accumulate carbohydrate instead of lipids. High levels of protein in *Chlorella pyrenoidosa and Chlorella vulgaris* biomass make them potential candidates for the formulation of fish feed.

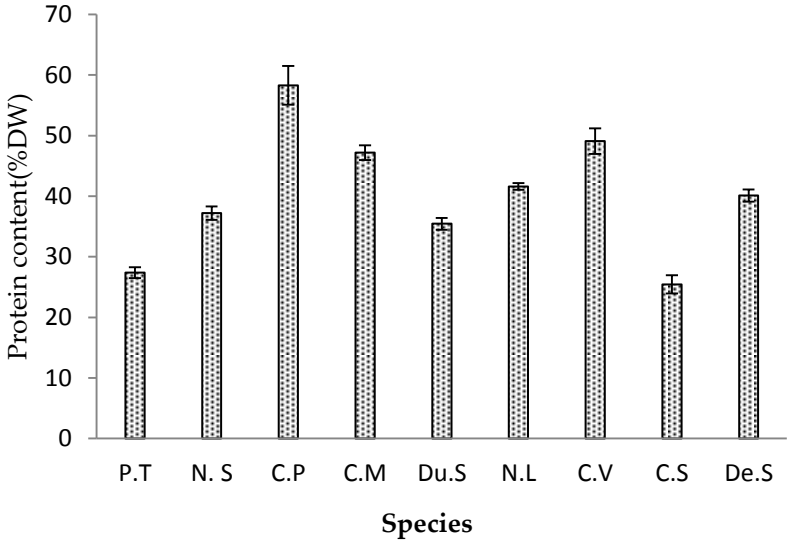


Figure 3.2. Total protein content in ICW-grown microalgae species. Samples of microalgae were harvested at the same growth stages (early stationary). Error bars show the standard deviations, n=2. *Chlorella sorokiniana* (C.S); *Phaeodactylum tricornutum (*P.T*); Dunaliella salina (*Du.S*); Nannochloropsis limnetica*(N.L)*; Desmodesmus* sp. (De.S)*; Nanochloropsis salina(N.S); Chlorella pyrenoidosa (C.P); Chlorella vulgaris* (C.V); *Chlorella minutissima* (C.M).

During the growth, the content of lipids and protein of microalgae varies as a function of culture age, growth medium composition and environmental factors (**Table 3.1**). In addition to the nitrogen concentration, phosphorus is another essential nutrient in growth media which promote the growth of microalgae cell and enhances the lipid formation. It has been claimed that N: P ratio in a growth media affect the lipid accumulation (He et al., 2013). A positive correlation between the protein content and concentration of nitrogen in growth medium was already reported by previous studies (Hu, 2003, Lv et al., 2010; He et al., 2013, and Guccione et al., 2014). Thus, the growth media directly influences synthesis of nitrogen-containing compounds such as protein, nucleic acids, amino acids, and chlorophylls depending on the levels of nitrogen (Griffiths et al., 2014). When protein is the target compound in the biomass, higher levels of nitrogen in the growth medium will be required. Use of an effluent like ICW decreases the production cost compared to cultivation with commercial growth media.

Table 3.1. Variations in total lipids and protein contents in *Chlorella pyrenoidosa* grown on 100% ICW. Values represented as % on dry weight basis± standard deviations, n=2. See paper 4 for more details.

Cultivation time (days)	**Lipids %DEW**	**Protein % DW**
4	5.75±0.1	53.1±1.2
8	8.20±0.2	58.2±3.5
11	7.87±0. 3	59.1±3.1
16	10.0±0.3	57.8±3.2

3.3.3. Fatty acid composition

The major fatty acids in the biomass of various microalgal species are presented in **Figure 3.3.** All samples were harvested at nearly the same growth stage. The fatty acid profiles varied between the classes. The growth condition and growth stage highly affects the fatty acid composition (**Figure 3.4**). The highest EPA (C20:5 n-3) was observed for Eustigmatophytes followed by diatom *Phaeodactylum* sp. Surprisingly, the biomass from microalgae *Chlorella minutissima* contained C22:5 n-3 and also C20:5 n-3, but other Chlorophyceae, including *Dunaliella* sp., *Chlorella sorokiniana, Chlorella vulgaris, Chlorella pyrenoidosa* and also *Desmodesmus* sp. contained no C20:5 n-3. The other LC PUFA, C22:6 n-3 was not observed in the samples, at considerable amounts. The main saturated fatty acid in all samples was C16:0. The contents of C16:1 n-7 was comparatively higher in eustigmatophytes and also in the diatom *Phaeodactylum* sp. For all Chlorophyceae microalgae, C18 fatty acids were dominant, but the profile was not the same, as reported previously (Volkman et al., 1989; Patil et al., 2007). The highest levels of linoleic acid C18:2 n-6 was observed in *Dunaliella salina.* For all of the Chlorella species as well as Desmodesmus sp., linolenic acid C18:3 was a major fatty acid in their fatty acid composition. The same results were reported by Patil et al. (2007). In addition to the environmental factors and growth media composition, the growth stage may influence the fatty acid composition. As an example, in **Figure 3.5** shows the effect of cultivation duration (culture age) on the content of total saturated (SAFA) and total n-3 unsaturated fatty acids in large scale cultivated *Nannochloropsis salina.* This information can determine the proper harvest time, e.g. to achieve the highest possible EPA so that the resulting biomass can be used in the formulation of aquatic feed as a source of EPA.

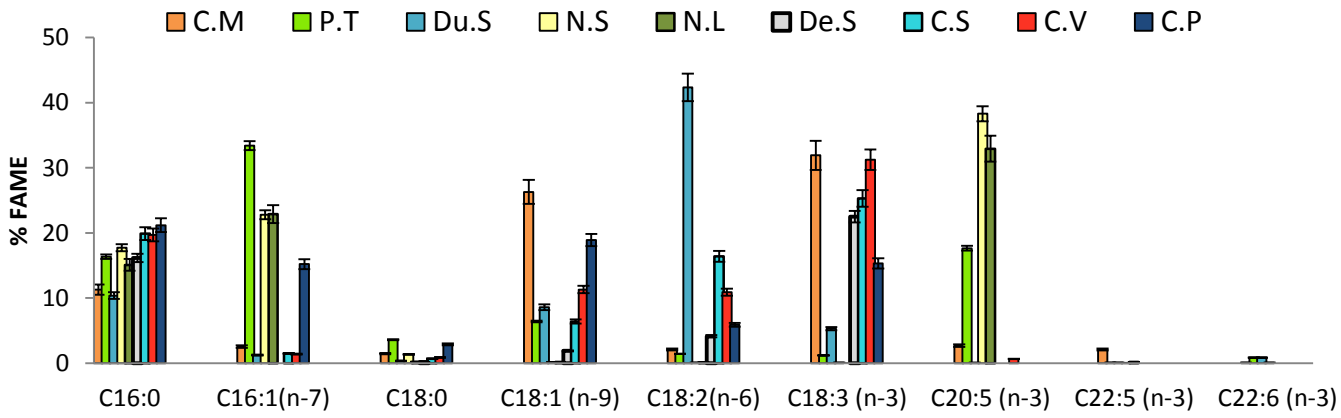


Figure 3.3. The typical fatty acid composition of waste-grown microalgae species. Samples of microalgae are harvested at the same growth stages (early stationary). Error bars show the standard deviations, n=2. *Chlorella sorokiniana* (C.S); *Phaeodactylum tricornutum* (P.T); *Dunaliella salina* (Du.S); *Nannochloropsis limnetica*(N.L); *Desmodesmus* sp. (De.S); *Nanochloropsis salina*(N.S); *Chlorella pyrenoidosa* (C.P); *Chlorella vulgaris* (C.V);*Chlorella minutissima* (C.M).

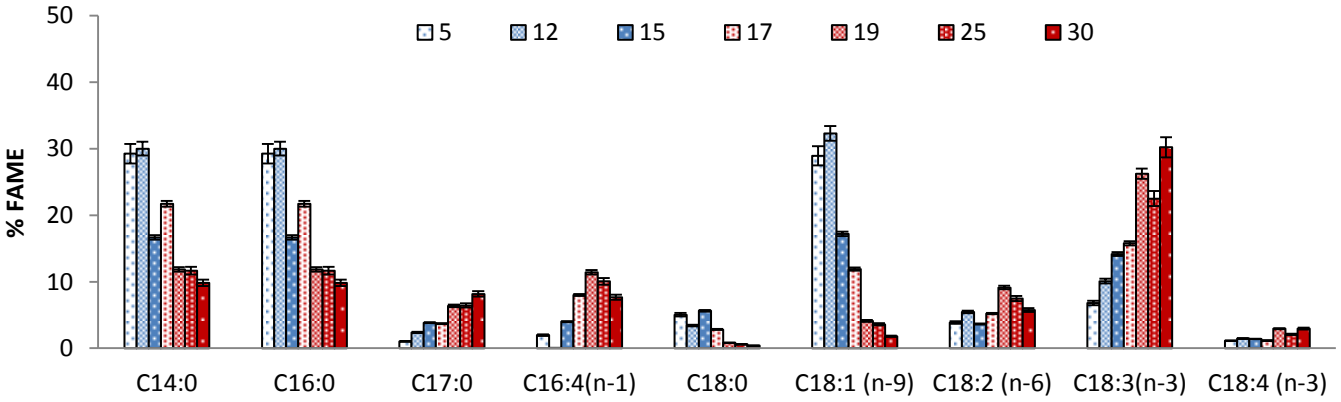


Figure 3.4. The variations in fatty acid composition of *Desmodesmus* sp., during the cultivation. The microalgae were grown on 100% ICW; details of cultivation experiments are presented in Chapter 4. Error bars show the standard deviations, n=2.

3.3.4. Amino acid composition

Amino acid composition in algae includes arginine, glutamine, lysine, asparagine and leucine as principal components (**Figure 3.6**). Amino acid composition in all microalgae is more or less similar (Guedes et al., 2015; Becker, 2007, Zhang et al., 2015), while can be influenced by variations in environmental parameters, culture age and also the growth medium composition.

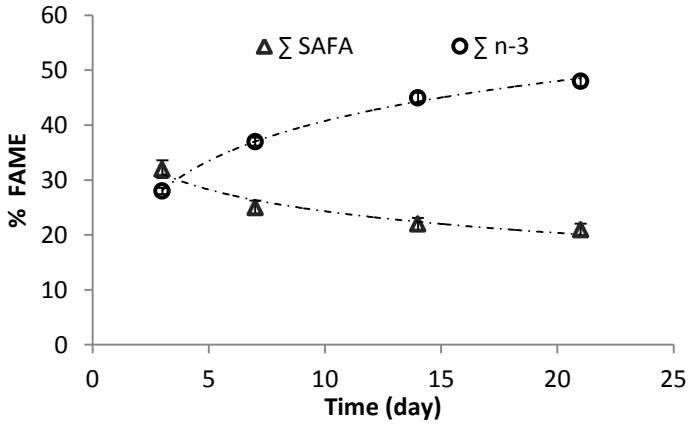


Figure 3.5. Variations in total saturated fatty acids (SAFA) and total n-3 fatty acid(n-3), In microalgae *Nannochloropsis salina* cultivated in 4000 L flat panel photobioreactor .details of cultivation condition are presented in paper 3 and also in Chapter 4. Error bars show the standard deviations, n=2.

The amino acid composition of *Chlorella vulgaris* and Chlorella *pyrenoidosa* included lysine, methionine, threonine, tryptophan, histidine, leucine, isoleucine, valine and phenylalanine (paper 4). During cultivation, contents of glutamine, asparagine and lysine declined (Figure 6). Total amino acid content also declined during the growth and particularly in stationary phase (**Figure 3.7**), which correlated with the variations in protein content in the biomass. The biomass from *Chlorella pyrenoidosa* included proper amounts of protein (ca. 58 % DW) and comparable amino acid composition, which make it a promising fish feed ingredient.

3.3.5. Natural antioxidants

In this project, the content and composition of natural antioxidants (phenolic compounds, carotenoids and tocopherols), as well as the antioxidative properties of the microalgae, have also been studied (Paper 1).

3.3.5.1.Carotenoids

The presence of antioxidants in fish feed is a fundamental requirement to prevent valuable LC PUFA and other sensitive compounds from oxidation induced losses and unwanted degradations. There is a global turning of the interests from synthetic antioxidants into the natural antioxidants such as phenolic compounds, tocopherols and carotenoids. Microalgae are known as a rich source of carotenoids (paper 1). The pigment profile of microalgae is identical for species of the same class and in some cases of the same family, so it can be used as fingerprinting of algae biomass as described in **Chapter 2**. However, the high carotenoid productivity for each species is achievable under suitable growth conditions. Green algae such as *Chlorella vulgaris, Chlorella pyrenoidosa, Chlorella sorokiniana* and

Desmodesmus sp., have shown a consistent growth with a good compatibility to the ICW as the main growth medium (papers 1 and 4). At the presence of carbon dioxide, in a nutrient-rich medium such as ICW, very high concentrations of valuable carotenoids (lutein) has produced by particular species (**Figure 3.8**), e.g. mutant *Chlorella vulgaris* as described in paper 4. Carotenoid productivity is not the same for all microalgae. In addition to the over expression of specific enzymes, additional storage space (outside of the photosystem) needs to be created inside the cell. In general, green microalgae such as *Desmodesmus* sp. and *Chlorella* sp. are suitable strains for lutein production when ICW is the main nutrient source.Thus, in addition to the species, growth medium composition and environmental conditions such as temperature and in particular light intensity influence the production of carotenoids (paper 1).

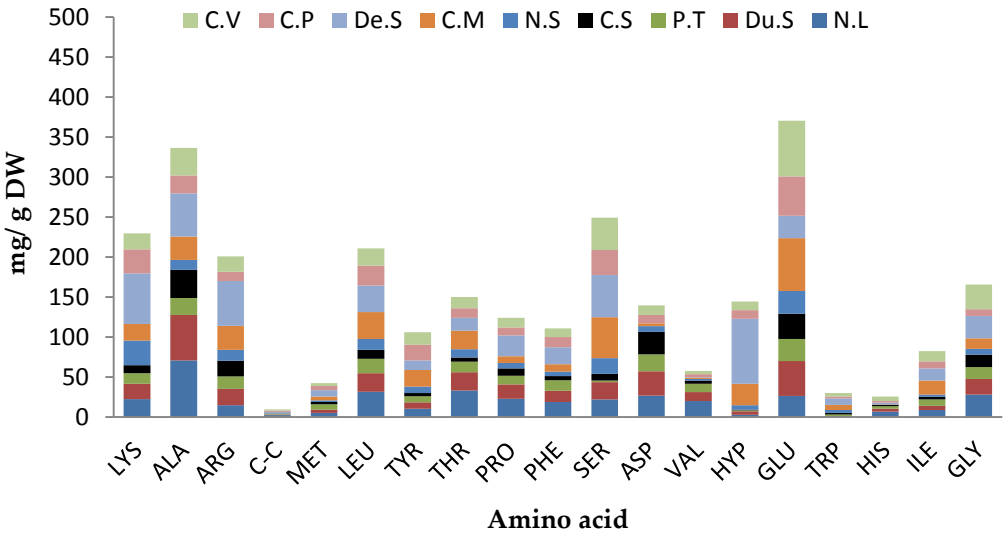


Figure 3.6. The amino acid composition of waste-grown microalgae species. Samples of microalgae are harvested at the same growth stages (early stationary). LYS; Lysine, ALA; alanine, ARG; arginine, LEU; leucine, MET; methionine, PHE; phenylalanine, PRO;proline, THR; threonine, TYR; tyrosine, ASP; asparagine, SER; serine, HYP; hesperidin, GLU; glutamine, VAL; valine, HIS; histidine, ILE; isoleucine, GLY; glycine.

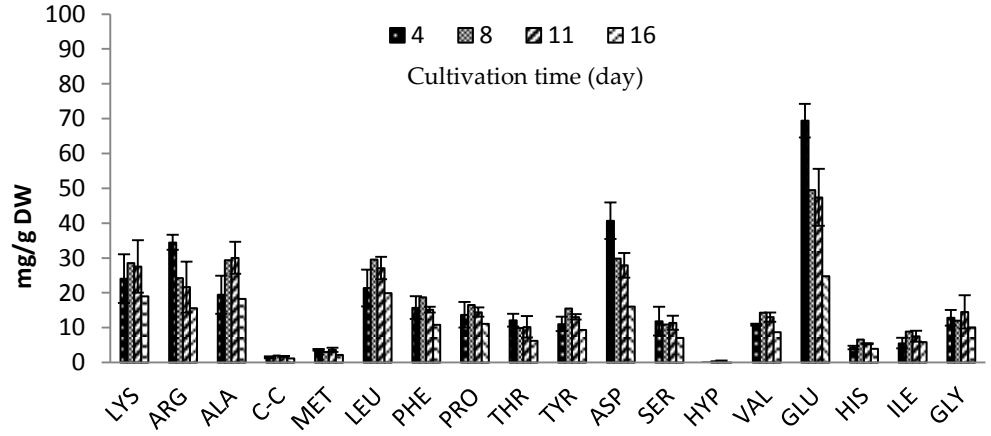


Figure 3.7. Variations in the amino acid composition of *Chlorella pyrenoidosa* cultivated on ICW as a main nutrient source (see paper 4 for more details). Error bars show the standard deviations, n=2. LYS; Lysine, ALA; alanine, ARG; arginine, LEU; leucine, MET; methionine, PHE; phenylalanine, PRO; proline, THR; threonine, TYR; tyrosine, ASP; asparagine, SER; serine, HYP; hesperidin, GLU; glutamine, VAL; valine, HIS; histidine, ILE; isoleucine, GLY; glycine.

Effects of culture age on the contents of carotenoids for *Nannochloropsis salina* (both in the laboratory and large scale); and *Chlorella* sp. (*Chlorella vulgaris* and *Chlorella pyrenoidosa*) have been demonstrated in papers 3 and 4, respectively. Carotenoids contributed to the 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging activity, ferrous reduction power (FRAP), and ABTS-radical scavenging capacity activity of microalgae biomass as it has shown in paper 1.

3.3.5.2. Tocopherols and phenolic compounds

Microalgae biomass mostly includes α-tocopherol, while β- and γ-tocopherols may also be synthesised by the cell, albeit in trace amounts. For all species which were studied in this project, contents of tocopherols increased by the culture age (papers 1, 3 and 4). The highest content of α-tocopherol was observed in large scale cultivated *Nannochloropsis salina* after 28 days of cultivation (**Figure 3.9**). The variations of tocopherols in microalgae species such as *Nannochloropsis salina*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris*, during the growth and in relation to the growth media were presented in papers 3 and 4. In paper six, the variation and the function of tocopherols as a natural antioxidant during the storage of dried algal biomass is presented. Accumulation of the tocopherols depended on culture age, growth medium composition and in particular nitrogen, light intensity and other environmental factors. The identified phenolic compounds varied from 10.07 ±0.04 μg/g for *Nannochloropsis salina* to 5.10 ±0.12 μg/g for *Phaeodactylum* sp. as shown in paper 1.

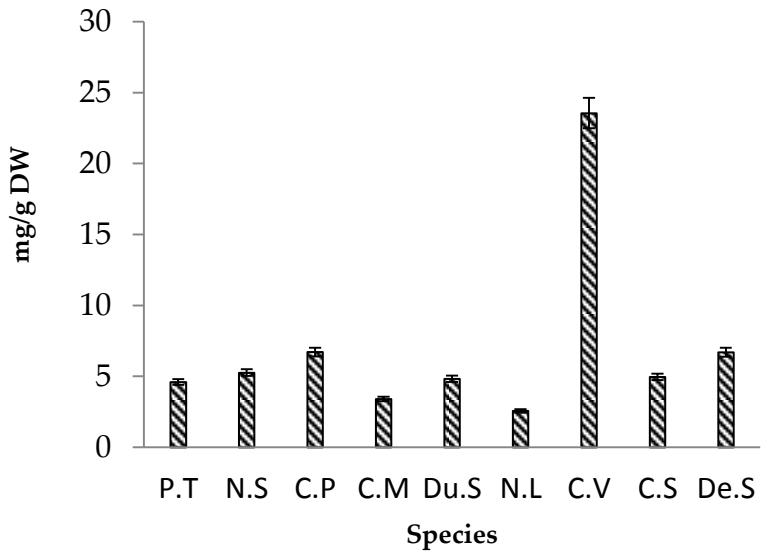


Figure 3.8. Total carotenoid contents in waste-grown microalgae species. The typical highest observed content of carotenoids for each species are presented. Error bars show the standard deviations, n=2. *Chlorella sorokiniana* (C.S); *Phaeodactylum tricornutum (*P.T*); Dunaliella salina (*Du.S*); Nannochloropsis limnetica*(N.L)*; Desmodesmus* sp. (De.S)*; Nanochloropsis salina(N.S); Chlorella pyrenoidosa (C.P); Chlorella vulgaris* (C.V); *Chlorella minutissima* (C.M).

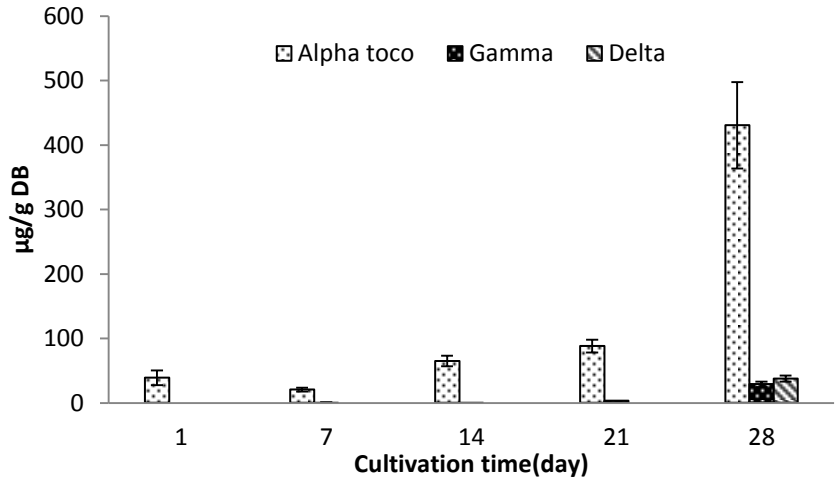


Figure 3.9. Variations in tocopherols (mostly α-tocopherol), In microalgae *Nannochloropsis salina* cultivated in 4000 L flat panel photobioreactor- Details of cultivation condition are presented in paper 3 and also in Chapter 4. Error bars show the standard deviations, n=2.

Only simple phenolic acids were identified by the method developed in this project, so the results did not include more complex phenolics. Identified phenolic acids included gallic acid, 2,5-dihydroxy benzoic acid, 3,4-dihydroxy benzoic acid, caffeic acid, ferulic acid, p-coumaric acid, salicylic acid, and cinnamic acid (paper 1). Phenolic compounds were found as the main contributors to the antioxidant properties of microalgae.

3.4. Selection of the microalgae species as a fish feed ingredient

Several criteria should be addressed in attempts to find proper microalgae as an ingredient in aquaculture feed. From a nutrition point of view, biomasses containing high protein, LC PUFA, and natural antioxidants are desired. The proper microalgae candidate(s) chosen for fish feeding should also be able to grow in the industrial process water and produce reasonable biomass. Therefore, the biomass production benefits from low cultivation price and also from valorization of the nutrients. The microalgal phycoremediation has been shown as a possible strategy by converting the nutrients to bioactive compounds such as protein, lipids, and pigments and saving of the waste treatment expenses. However, the proper harvest time shall be considered to guarantee the effectiveness of nutrient removal, biomass productivity and appropriate biochemical composition. The microalgae *Chlorella pyrenoidosa* represented biomass including very high protein content as well as promising amino acid and carotenoid composition while lacking the LC PUFA. A high EPA biomass from *Nannochloropsis salina* can be used as a source of LC PUFA.

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Chapter 4; Cultivation of microalgae on industrial process water as nutrient source

4.1. Part one; Laboratory experiments for the selection of microalgae species

4. 1.1. Introduction

Microalgae contribute to approximately 50% of global photosynthetic activity. Autotrophic algae convert solar energy and CO_2 absorbed by chloroplasts and used in respiration to produce required energy for growth and reproduction. Microalgae contribute to nearly 50% of the world's photosynthetic activity (Chiu et al., 2015). In recent years, the eligibility of using microalgae for the bio-utilization of atmospheric CO_2 through photosynthesis has been demonstrated in literature as a potential way to reduce the carbon dioxide emissions and convert it to valuable biomass, which could be used in various applications (Ho et al., 2011). Some microalgae have proven to have the capability to grow on waste water (rural, agricultural or industrial) as a nutrient source. Cultivation of microalgae on waste water reduces its biological oxygen demand (BOD), chemical oxygen demand (COD) through bio-utilisation of the chemical compounds such as ammonia, nitrate, phosphate and heavy metals (Chiu et al., 2015), which are typically present in all waste waters and difficult to remove. Therefore, the large-scale cultivation of microalgae can be applied as the last step (also known as a tertiary process) of the treatment in wastewater treatment systems (Martinez et al., 1999). Microalgae can use inorganic nitrogen and phosphorus for their growth (papers 3 and 4). However, they could also remove heavy metals and toxic organic compounds (e.g. phenols) which are found in some wastewaters (e.g. agricultural waste waters). On the other hand, above mentioned toxic compounds accumulate in the microalgae body, so that the application of resulting biomass is limited to those such as chemicals and biofuel. Hence, the application of waste grown microalgae as a fish feed ingredient is limited to those cultivated on particular wastes containing no toxic compounds.

Anaerobic treatment is a biological waste disposal technique in which, a microbial consortium (methanogenic bacteria) reduce the polluting load of waste water by conversion of organic compounds to the biogas. This technology is indicated as one of the globally most favoured waste treatment methods (Mc Hugh et al., 2003). The conversion process happens in an anaerobic sludge tower reactor with internal circulation (ICT) which contains methanogenic microbial microorganisms. The resulting methanogenic biogas consists of methane (50–80%), CO_2 (20–50%), volatile ammonia, hydrogen sulphide, and hydrogen. The methanogenesis process also produces a dilute effluent (so-called water from IC tower; ICW). This effluent include ammonia (but no nitrite or nitrate), and inorganic phosphorus as the main components as well as secondary compounds such as iron and sulphate (paper 3; Mc Hugh et al. 2003). In this project we used industrial process water, which was collected from Novozymes plant (Kalundborg, Denmark) and treated as described above and

therefore it will be termed (ICW) in this thesis. The chemical composition of ICW is shown in **Table 4.1**.

The effluent stream from biogas plants typically include high concentrations of nitrogen (e.g. 3800 mg/L) and phosphorus (e.g. 500 mg/L) but no organic carbon (Ruiz-Martinez et al., 2011; Arnold, 2013). The individual effluent streams which were used in our project contained lower concentrations of nutrient and were free from toxic compounds and heavy metals, representing a promising growth medium for cultivation of autotrophic microalgae for biomass production. The effluents like this would typically require pretreatment (filtering-sterilization) before they could be used in algal cultivation (Arnold ,.

Table 4.1. The chemical composition of industrial wastewater which was used as main nutrient source.

Item	Unit	Amount
pH	-	8.1
Suspended solids	mg/L	20
Total N	mg/L	190
Ammonia + ammonium-N	mg/L	150
Nitrite + nitrate	mg/L	<0.1
Total P	mg/L	11
Sulphate	mg/L	3.6
Total cyanide	µg/L	2.5
Total Alkalinity	mmol/L	62.5
EDTA	mg/L	<0.5
Sodium(Na)	mg/L	1500
Cadmium (Cd)	µg/L	<0.05
Copper (Cu)	µg/L	3.4
Iron (Fe)	mg/l	0.23
Cobolt (Co)	µg/L	<0.5

Batches of ICW were filtered using an out-side-in dynamic cross-flow microfiltration Bio-Booster system Microfiltration by 0.2 µm ceramic disc filters and stored at -20℃ until use. Pre-treatment of ICW was done by dynamic cross-flow microfiltration system (Grundfos A/S; Bjerringbro, Denmark). This unit (**Figure 4.1**) represented a compact solution, very easy to operate and with a low energy consumption. The unit was equipped with a semi-automatic chemical enhanced backwash (CEB)

system but does not allow back-pulse for additional preventive cleaning of the membrane (E4W report).

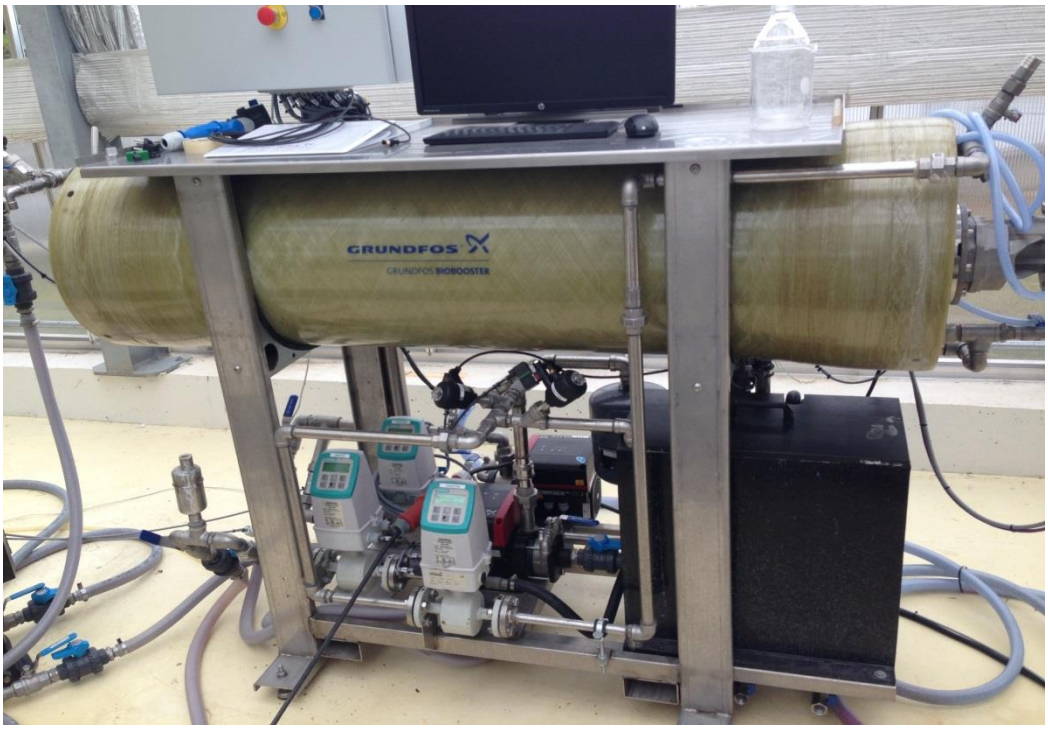

Figure 4.1. The Grundfos mini microfiltration unit (mini-MFU), representing the dynamic cross-flow filtration set-up tested for ICW microfiltration. **Specifications:** System volume: 200 L, capacity: 100 – 1000 L/h (depended on media), operation pressure: Max 2.5 bar, membrane area: 6.84 m², membrane pore size: 200 nm, membrane material: Aluminum oxide.

4.1.2. Screening and selection of microalgae

An algal strain suitable for aqua feed applications should be able to grow on ICW with ability to produce reasonable biomass rich in protein, PUFA fatty acids and carotenoids, in order of importance. The good strain should also be easy to cultivate and harvest.

Various autotroph species including *Chlorella pyrenoidosa* (*A*TCC®75668™), *Chlorella sorokiniana* (CCAP 211/8K), *Chlorella vulgaris* (SAG 211-81), *Nanochloropsis salina* (SAG: 40.85), *Nannochloropsis limnetica* (*SAG*18.99), *Desmodesmus* sp. (wild type, isolated from waste water treatment system, Kalundborg,

Denmark), and *Arthrospira plantaesis* (SAG 85.79), *Chlorella minutissima (NIVA-CHL 183)* were cultivated to evaluate their ability to grow on ICW. Cultivation was done at laboratory (0.5- 5 L), pilot scale (10-100 L) and large scale, for selected microalgae species. (See papers 1, 3 and 4 for more details). In **Figure 4.2**, cultivation set ups which were used during the project is shown.

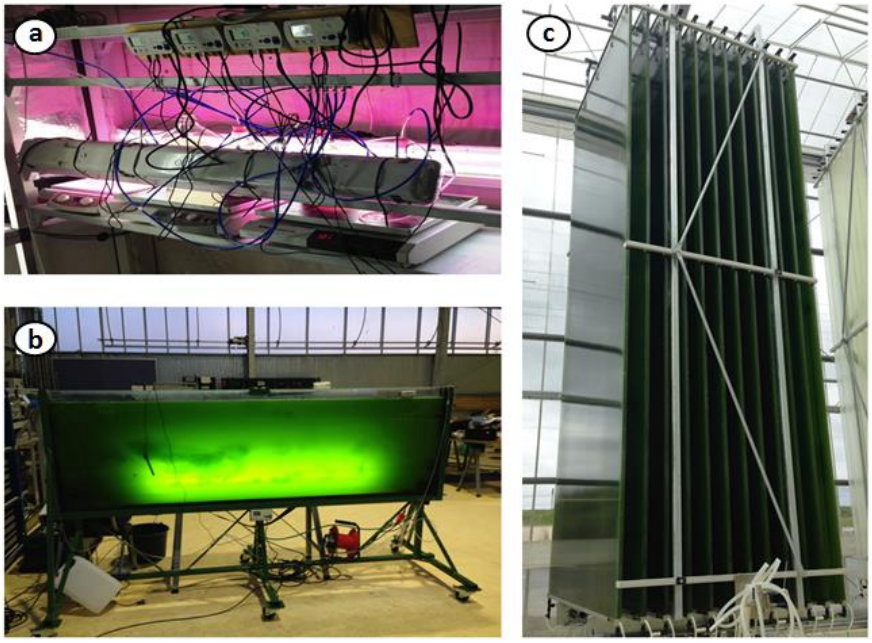

Figure 4.2. Experimental cultivation set up at the: (a) laboratory; (b) pilot and; (c) large scales. See papers 1, 3 and 4 for full details.

Nitrogen which is highly required for microalgae growth accounts a major energy burden as 45% of the total energy input so that the use of an effluent stream containing nitrogen will lower the energy burden and improve the overall sustainability of microalgae cultivation and the resulting biomass (Gutierrez et al., 2016). The main inorganic nitrogen forms in microalgae growth media include ammonia-ammonium, nitrate and urea. As the main component in ICW is ammonia, the microalgal growth, biomass productivity and the composition of biomass during the growth highly depend on the ammonia tolerance and the ability of the algae to uptake and utilise ammonia as the (main) nitrogen source. It has been found that chemical forms of nitrogen influence the structure of phytoplanktons communities in lakes. As an example, it has been demonstrated that algal blooms by diatoms and flagellates/dinoflagellates happened in the lakes where the main nitrogen form was nitrite and ammonia, respectively (Collos and Harrison, 2014). Keller et al. (1987) reported that concentrations of 100 μM ammonium were toxic for 200 species of marine phytoplankton

communities. Ammonium toxicity in water can be due to the effects of both the unionised ammonia (NH_3) and the ionised ammonium. Ammonia is considered to be the most toxic form because it is uncharged and lipid soluble and easily diffuses across cell membranes. In contrast to ammonia, the charge on the membrane hinders the passage of the charged ammonium ion. No chemical method can measure these two forms separately. Present chemical methods measure both forms as "ammonia + ammonium". The relative concentration of each form is strongly dependent on pH and to some lower extent on temperature, while the effect of salinity is minor. However, the ammonia tolerance level is highly species dependent. Collos and Harrison (2014) ranked various classes of microalgae as Chlorophyceae > Cyanophyceae, Dinophyceae, Diatomophyceae, and Raphidophyceae, based on the tolerance to the ammonia toxicity. Various microalgae species were cultivated on different percentages of ICW as main nutrient source, or a combination of ICW and standard reference growth medium (**Figure 4.3** and **Figure 4.4**). The most tolerant species were found to be *Chlorella pyrenoidosa* (**Figure 4.4a**), *Chlorella vulgaris* (**Figure 4.4b**), *Desmodesmus* sp. (**Figure 4.3a**), *Chlorella minutissima* (**Figure 4.3d**), and *Chlorella sorokiniana* (data are not shown here), all from Chlorophyte. For both *Nannochloropsis salina* (**Figure4.3c**) and *Nannochloropsis limnetica* (eustigmatophyte), our observation demonstrated that substitution of more than 20% of standard F/2 growth medium retards the growth. On the other hand, none of these *Nannochloropsis* species grew properly on ICW as the main nutrient source, even on fivefold diluted ICW (data are not shown here). However, 25% substitution of F/2 growth medium with ICW has no significant effect on the microalgal growth. The cyanobacterium *Arthrospira platensis* (**Figure 4.3b**), was not able to grow on ICW only. However, when 25% of spirulina growth medium, was substituted with ICW, the growth improved significantly (**Figure 4.3b**). In general and for all laboratory growth experiments, the algae growth start was retarded for the algae cultivated on higher percentages of ICW (e.g. 75% and 100% ICW). In general, microalgae from the class chlorophtye grew well on two third or half diluted ICW as the main, unique nutrient source and produce promising amounts ofbiomass (**Figure 4a and 4b**). On the other hand, both *Chlorella pyrenoidosa and Desmodesmus* could exclusively grow on 100% ICW (**Figure 4a** and **3a**, respectively), which is an important finding when the water resource is a limitation and also for valorization of the nutrients to reduce the BOD and COD values.

4.1.3. Nutrient valorization in ICW grown microalgae

The efficient phycoremediation by microalgae such as *Chlorella* sp. was reported previously in several studies (Tam et al., 1989; Liu et al., 2016; Chiu et al., 2014).

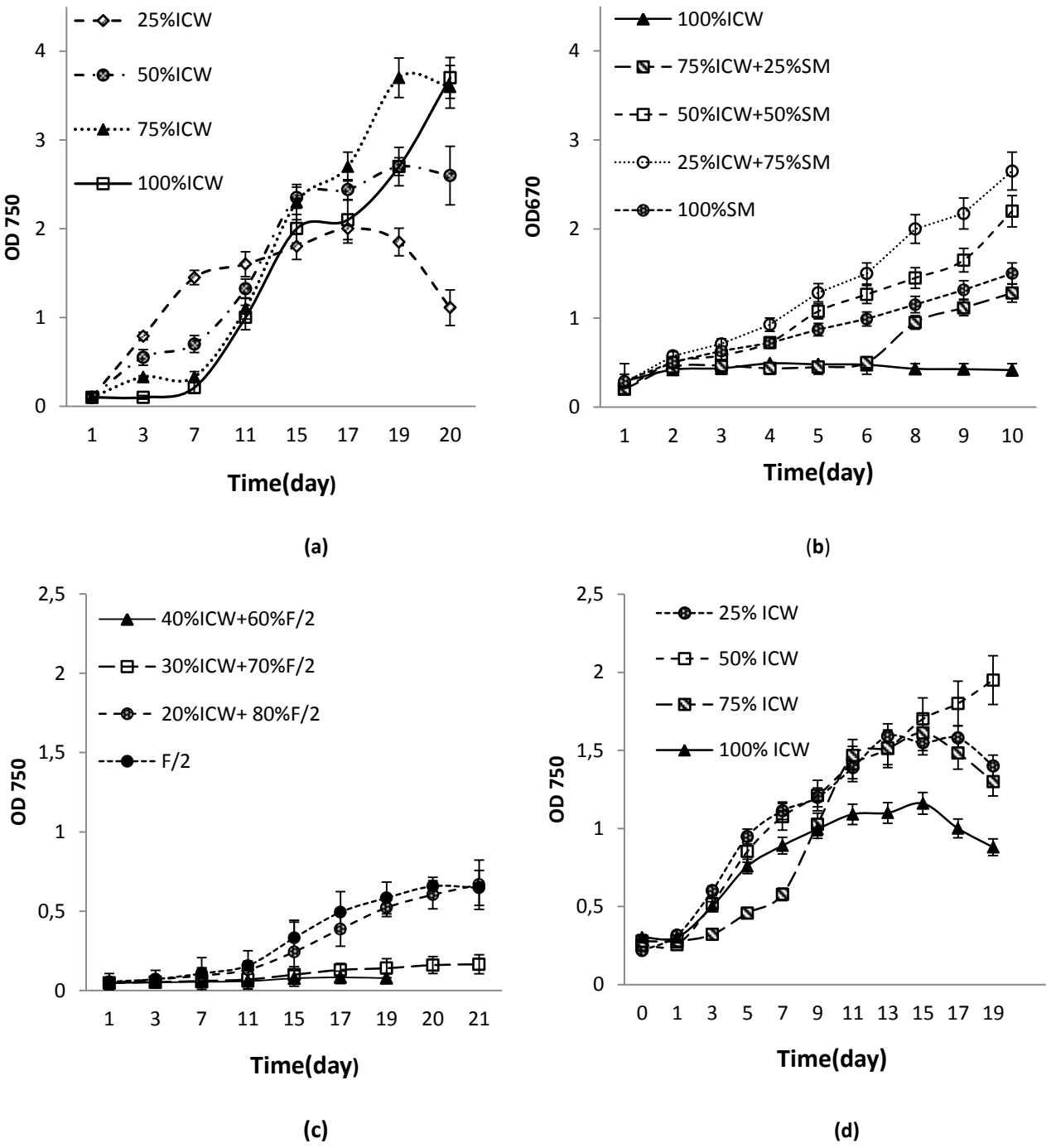

Figure 4.3. Effect of different levels of industrial process water on growth of various microalgae species, **(a)**; Desmodesmus sp.**, (b);** *Arthrospira plantesis,* **(c)**; *Nannochloropsis salina* and **(d)**; *Chlorella minutissima.* Error bars show the standard deviations (n=2). SM; spirulina medium as in Aiba and Ogawa (1977). F/2; the standard growth medium (as in paper 3)

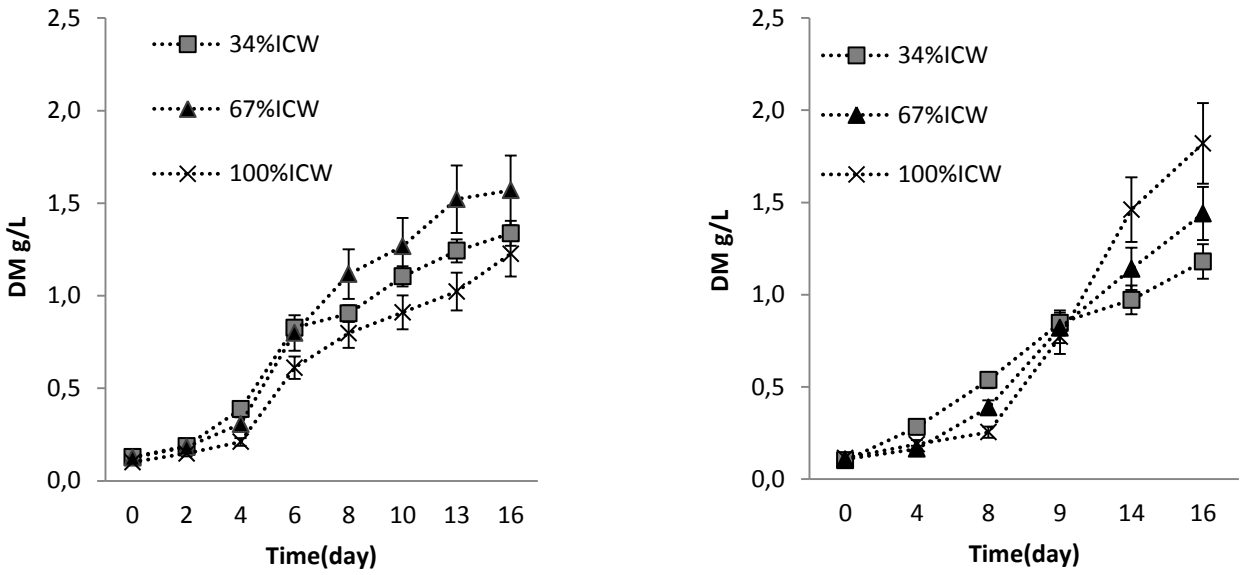

Figure 4.4. Effect of different percentages of industrial process water on growth of **(a);** *Chlorella vulgaris* and**(b);** *Chlorella pyrenoidosa* Error bars show the standard deviations (n=2). See paper 4 for more details.

These reports suggest phycoremediation as a feasible strategy to reduce the released amounts of inorganic (and organic) nutrients into natural waters, and valorize these resources by converting the nutrients to bioactive compounds such as protein, lipids, and pigments. The microalgal valorization also include carbon dioxide(CO_2) bioutilization, owing to their CO_2 sequestration ability. Carbon dioxide is another by-product of methanogenic anaerobic waste treatment process. For all of the cultivation experiments at the laboratory, pilot and large scales, an automatically controlled air stream containing 5% CO_2 was used as dual action, as a carbon source and also as a pH regulation agent. For the algae grown under CO_2, significant improvements in the growth rate and biomass productivity were observed. Biomass generation of microalgae also has potential benefits in cleaning the environment, (Singh et al., 2012). It is estimated that one kg of dry algal biomass utilizes about 1.83 kg of CO_2 (Gupta et al., 2015).

4.1.3.1. Nutrient uptake

Both *Chlorella pyrenoidosa* and *Desmodesmus*, which were able to grow on 100% ICW, tested for the ability of uptaking main nutrients as ammonia+ammonium and phosphorous (**Table 4.2**). The *Chlorella pyrenoidosa* was large scale-cultivated (October-December 2015) in a tubular photo bioreactor

(see next section for details of the PBR), while *Desmodesmus* sp. was cultivated at laboratory scale (20L), 20 L Schott bottle, 20±5℃, under artificial light; 100 μmol p sq. m^{-1} (Paper 1). In both experiments, microalgae efficiently reduced the nutrients of ICW, so that the polluting load of the effluent declined by more than 90%. Microalgae cultivation on ICW stream reduces the energy input from nitrogen as a nutrient to support cell biosynthesis, which can be indicated as an efficient energy input reduction strategy. Using the ICW either as the main or substituting growth medium improves the chemical composition of microalgae (see Paper 3 and 4).

Table 4.2. Valorization of ammonia and phosphorus for, (**a**); *Chlorella pyrenoidosa* and (**b**); *Desmodesmus* sp. cultivated on 100% ICW. All values are presented as in mg/L.

Time(day)	Ammonia ($NH_4^+ - N$)*	Phosphate ($PO_4^{-3} - P$) *
0	206±2.0	2.74±0.2
21	0.017±0.0	0.029±0.0

(**a**)

Time(day)	Ammonia ($NH_4^+ - N$)*	Phosphate ($PO_4^{-3} - P$) *
0	190±2.6	6.12±0.5
12	0.077±0.0	0.05±0.0

(**b**)

*All values are demonstrated in mg/L

4.1.4. Conclusion for part one

Various microalgae species were evaluated for their ability to grow on ICW. Chlorophyte could grow on ICW as main and unique nutrient source, and uptake nutrient, so that the environmental pollution load of ICW was efficiently decreased. For eustigmatophyte and cyanobacteria, partial substitution of standard growth medium with ICW to levels less than 25% showed no adverse effect (eustigmatophyte) or even improved the growth (cyanobacteria)compared to the algae cultivated on standard growth media as a reference. Two microalgae species including *Nannochloropsis salina* and *Chlorella pyrenoidosa* were selected for large scale experiments due to their nutritive composition (Chapter 3, papers 3 and 4), and also for the ability to grow on a growth medium containing ICW.

4.2. Part two; large scale cultivation of microalgae

4.2.1 Introduction

Currently, there is much interest in cultivating microalgae as feedstock for the production of bioactive compounds and also as alternative ingredients in the aquatic feed (Zittelli et al., 2013). Industrial production of microalgae for the applications such as feed and biofuel is currently non-economical. Among several factors, those connected with large-scale cultivation are of the greatest importance (Zittelli et al., 2013). Large scale cultivation methods can be divided to (photo bioreactors) PBR and open ponds. The mass production of algal requires high energy expenditures (mixing, cooling, and embodied energy). Open ponds have a more favourable energy balance, but cultures in large-scale open ponds have a significant water evaporation rate. On the other hand, keeping the quality and purity of the biomass during the cultivation is difficult (Gupta et al., 2015), even impossible. During the last few years, several new PBR designs have been presented mostly aimed at reducing the overall expense, and to improve the biomass productivity. The cultivation reactors are divided into two main groups as open and closed systems (Gupta et al., 2015).

4.2.1.1. Open systems

The open cultivation systems include large shallow ponds, open vessel (made from plastic, cement or metals), ponds (circular ponds and raceway ponds). Open ponds are straightforward and cheap algae cultivation system, resembling artificial lagoons and oxidation ponds in wastewater treatment (Sharma et al., 2013). Most of the current open ponds are designed mostly based on the raceway pond idea first introduced by Oswald (1969). They are cost effective, simple and flexible, easy to construct and operate, and suitable for mass cultivation. Disadvantages of this system are indicated as high water evaporation rate, gas (e.g. O_2 and CO_2) exchange, and high required space. However, the control of temperature and light in this system is not possible so that the biomass productivity is low. A major disadvantage is also that the culture purity control is impossible.

4.2.1.2. Closed systems

Closed systems (also known as photobioreactors) include closed vessel, tubular (either horizontal or vertical) and flat panel PBRs (FP PBR).

The stirred vessel PBR benefits from good heat and mass transfer which is provided by mechanical stirring (e.g. paddles) and/or gas injection spurge (Gupta et al., 2015). On the other hand, mechanical agitation increases the overall process energy so the system volume is limited. In general, 20-30% of total vessel volume should be free as head space. The simplest examples of stirred vessel PBRs are the glass flasks and plastic bags which are widely used in laboratory cultivation experiments (**Figure 4.5a** and **b**). For large scale reactors, the stirring efficiency should be high enough to guarantee the nutrients and light availability and temperature homogeneity, which requires a relatively large input of energy per unit volume. However, the stirring mechanism should be done in such a way that

foaming, warming up and also too much vortexing is avoided. Therefore, the special design of paddles and also using the baffles are critically required.

Narrow light path-length PBRs includes either **horizontal** or **vertical** designs. These types of PBRs are made from glass (small-medium scales) or transparent polymers (large scale) and are circulated by the gas stream, either by pump or airlift mechanism. The **airlift mechanism** relies merely on the physical separation of gas from the liquid (culture) in the PBRs. Two main streams are known as rising, and falling streams form the continual flow of the fluid. The sparging gas is penetrating through the raising stream moves it upward and then leaves the stream at the top head space (of each tube or the whole system). Then the gas free stream falls, forming a continuous circulating-mixing pattern (Gupta et al., 2015). This technology is used at tubular or flat panel PBRs. The **horizontal tubular PBRs** (**Figure 4.6**) are suitable for outdoor algae cultivation (Gupta et al., 2015). Their mixing efficiency is appropriate, and the static pressure is low. In general, tubes have diameters of only 10-60 mm, while the total length of the system could be several hundreds of meter. The efficient cultivation requires careful calculation and proper design to guarantee a continuous gentle-uniform flow through the rest of system, to minimize fouling. The surface to volume ratio in this system is very high, which is a benefit. However, it can also increase the system temperature. In such cases, cooling of the culture is required, which increases the overall required energy.

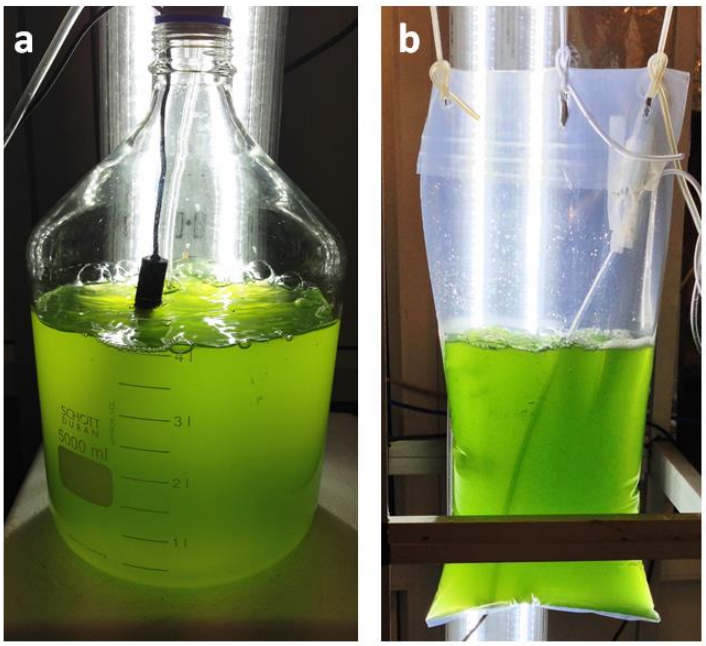

Figure 4.5. Laboratory scale closed vessel PBRs. Bottle (**a**) and 10 L plastic bag (**b**). The capacity of plastic bags depends on the species and growth conditions. Agitation is performed by gas sparge (plastic bags), or both gases sparge and shaking/stirring (flasks).

Another disadvantage of the system is that dissolved oxygen builds up which may cause photoinhibition (Gupta et al., 2015).

Vertical PBRs are sub-divided to the **bubble columns** and **airlift PBRs.** In bubble columns, the sparge gas (air and CO_2), perform the mixing and the agitation. The photosynthetic efficiency highly depends on gas flow rate because the liquid is regularly circulated from the tank to outer tubes at a higher gas flow rates (Barbosa et al., 2003). Bubble column PBRs have many advantages, including low capital cost, high surface area to volume ratio, good heat and mass transfer and satisfactory release of gases (CO_2 and oxygen) from the liquid.

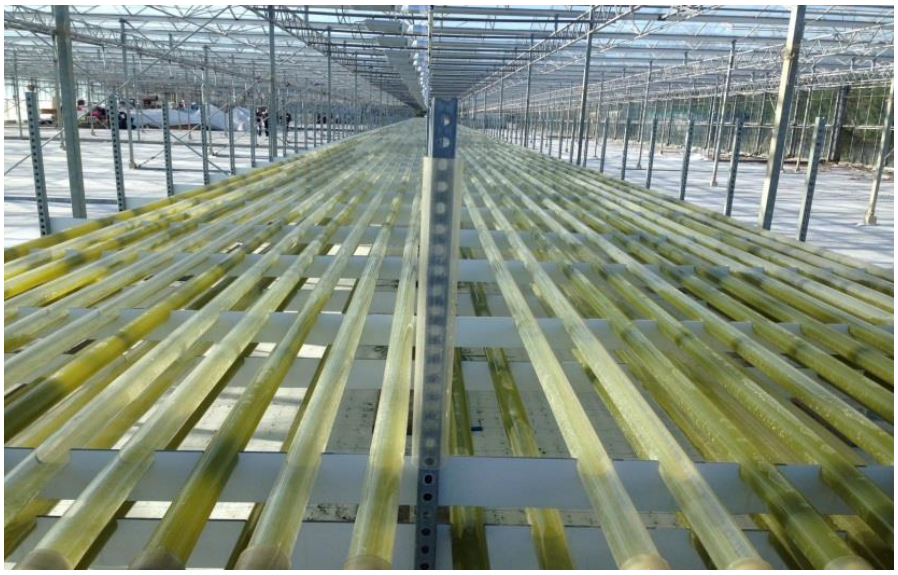

Figure 4.6. The two-phase horizontal tubular PBRs system GemTubeTMMK(500 m^2), from LGEM, the Netherland. The system is being successfully used for the production of *Nannochloropsis* sp.

The **vertical airlift PBRs** (either **tubular** of **flat panel**, (**Figure 4.7**), benefits from simple geometry and short light path length (less than 30 mm for FP PBRs, and 10-60 mm for T PBRs) which increases the light absorption efficiency (Tredici and Zittelli, 1998). Flat panel PBRs consist of several transparent plates which are installed on the mainframe faced to the light source. The circulation of algae suspension can be done using a pump or by the air stream.

In FP PBRs, the surface to volume ratio is very high. Each panel is divided into small channels. Moreover, the gas exchange and degassing of the culture is performed by bubbling air from the bottom of each channel. Compared to horizontal PBRs, the risk of accumulation of dissolved O_2 in flat-plate PBRs is relatively lower. Due to the shorter light path length, the biomass productivity achieved

by cultures in flat-panel PBRs is lower than tubular systems, due to lower light dilution (Tredici and Zittelli, 1998).

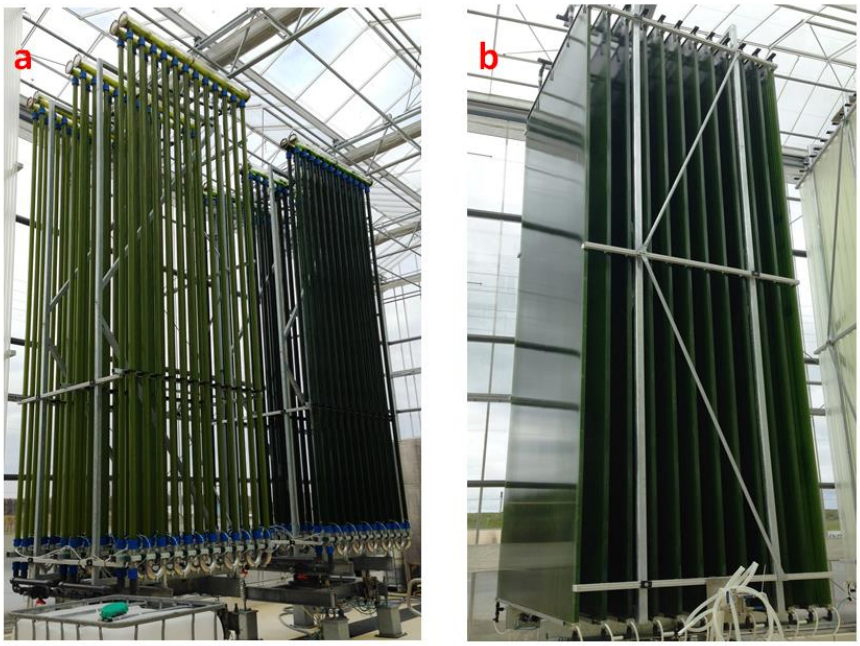

Figure 4.7. The 1500 L airlift vertical-tubular PBR(**a**); and 4000 L flat panel PBR(**b**) from ecoduna produktions-GmbH(Bruck ander Leitha, Austria). Both systems were installed in kalundborg microalgae facility(Kalundborg, Denmark), and were used for large scale cultivation trials *Nannochloropsis salina*(paper 2), and *Chlorella pyrenoidosa*(paper 3).

On the other hand, FP PBRs require automated temperature and pH control systems. Moreover, the hydrodynamic stress resulting from aeration is higher than T PBRs, so the system could not be used for some algal strains, e.g. *Arthruspira* sp. *and Dunaliella* sp.

4.2.2. Large scale cultivation of selected microalgae

4.2.2.1. Cultivation of *Nannochlorosis salina in* 4000 L FP PBR

Large scale cultivation experiments were successfully performed at the Kalundborg microalgae facility using 4000 L flat panel PBR system, type hanging garden (**Figure 4.7b**), as described in paper 3. The photo-bioreactors were designed to track the sun thereby allowing the algae to grow most efficiently in natural sunlight. Each FP PBR consisted of 12 hollow chamber sheets (width 2100 mm; height 5600 mm and depth 32 mm) which were installed on the main frame (**Figure 4.7b**). The microalgae suspension pumped into the sheets and then continuously circulated through the module. The nutrient stream was transferred to the sheets by hydrostatic pressure and then was distributed

continuously using air lift effect. In these situation surface hit by sunlight is multiplied and the light is being distributed so the irradiation does not increase the temperature. All of the sheets were connected via a hose system thus ensuring the obstruction-free transfer of the nutrient solution. The algae growth rate in winter conditions was slow, but the nutritional properties of the biomass were improved compared to laboratory-scale trials (paper 3). EPA reached 44.2% ± 2.30% (of total fatty acids), and α-tocopherol reached 431±28 μg/g of biomass (on dry weight basis) after 21 days of cultivation. Variations in chemical compositions of *Nannochloropsis salina* were studied during the cultivation (paper 3). The culture remained pure and grew during six months of cultivation (Autumn 2015- Spring 2016). The static pressure has not proven to have any adverse effects on the microalgae during the cultivation so that *Nannochloropsis salina* could be represented as a candidate for large-scale cultivation by FP PBR.

4.2.2.2. Cultivation of *Chlorella pyrenoidosa* in 1500 L tubular PBR

A large-scale cultivation experiment was done for *Chlorella pyrenoidosa* in Kalundborg microalgae facility. Data from lab scale experiments were used for the experiment (paper 4). Cultivation unit was a 1500 L tubular PBR (**Figure 4.7a**), and the cultivation was started in February 2016. The inoculum was prepared previously in pilot scale 130 L flat panel PBR (**Figure 4.2b**) and then transferred to the PBR. ICW(100%) was used as growth medium. No artificial light was used during the experiment. Recultivation was performed every 20 days. During 60 days of cultivation, the dry matter has reached 6.15 g/L (**Figure 4.8**).

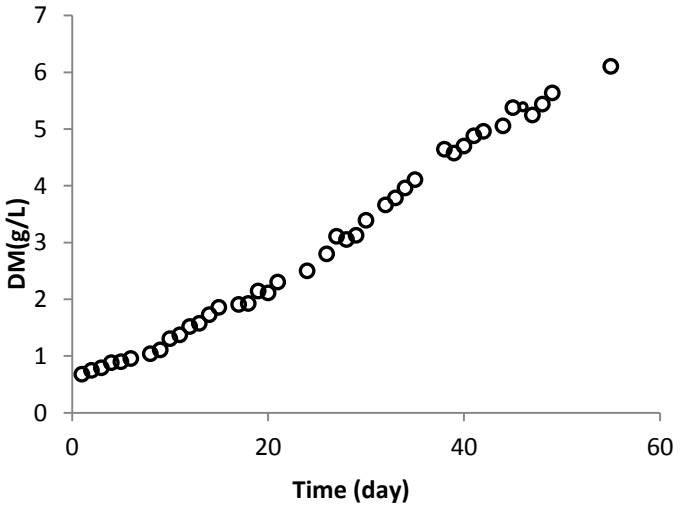

Figure 4.8. Variations in dry matter(g/L) during the cultivation experiment. Cultivation was performed in a 1500 L T PBR from ecoduna. The optical density(OD 750 nm)data converted to dry matter (paper 4 for details). The culture temperature ranged from 12 to 20 ℃ during cultivation.

The final biomass composition included 58.3±1.0% protein and 10.1±0.2% lipids. The cultivation was stopped after 3 months due to the severe contamination by Ciliates (**Figure 4.9**), which caused a sudden sedimentation resulted in reactor fouling.

Figure 4.9. Microscopic observation (X 100) of the *Chlorella pyrenoidosa* culture contaminated by protozoa (Celiates).

4.2.3. Conclusion for part two

Two microalgae species including *Nannochloropsis salina* and *Chlorella pyrenoidosa* were cultivated in large scale using FT PBR and TPBR, respectively. *Nannochloropsis salina* growth rate at winter was slow, but the nutritious composition of the large scale cultivated algae biomass was improved compared to the laboratory experiments. Cultivation of microalgae *Chlorella pyrenoidosa* on 100% ICW at TP PBR was successful but interrupted by the grazers. The chemical composition of the biomass resembled the results from laboratory experiments. The biomass of both species can be used as a promising fish feed ingredient (Paper 3 and 4).

4.3. References

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Chapter 5; Downstream processing of process water grown microalgae for production of fish feed ingredient

5.1 Introduction

This study was aiming at designing a downstream process for the economical production of high-quality biomass from microalgae grown on industrial process water so that it can be used as a fish meal ingredient. Target biomass is supposed to have high protein content (50-60% DW), moderate lipid contents (10-18% DW), and elevated levels of bioactive compounds such as carotenoids, tocopherols, essential amino acids and long chain polyunsaturated fatty acids. The downstream process methods is itemised to harvesting, up-concentration, drying, and a heat treatment process for enzyme inactivation. Cell rupture and further extraction of pigments or lipids was omitted. Microalgal carotenoids and lipids (with high contents of polyunsaturated fatty acids) can add value to the final product. This chapter represented the processing set up which developed for the production of microalgae biomass as a fish feed ingredient . In paper 5 various downstream processing technologies applied to microalgae with a focus on microalgae as a fish feed ingredient is reviewed.

5.2. Materials and Methods

During the project, and through a screening process, several microalgae species including *Nannochloropsis limnetia*, *Nannochloropsis salina, Chlorella vulgaris, Chlorella sorokiniana, Chlorella minutissima, Chlorella pyrenoidosa, Monodopsis subterranea, Phaeodactylum tricornutom, Dunaliella salina* and *Desmodesmus* sp. have been cultivated on ICW as described in Chapter 3 and 4. Microalgae were evaluated for the growth performance, biomass productivity, protein, lipid, fatty acids, pigments, amino acids composition and natural antioxidants. Two microalgae, with high protein (*Chlorella pyrenoidosa)*, and high EPA (*Nannochloropsis salina*) content were selected and cultivated in industrial scale in Kalundborg microalgae facility(Chapter 4). All of the mentioned species have been used in this chapter. A large scale cultivated algal biomass from *Nannochloropsis salina* was used for drying experiments.

5.2.1 Analytical methods

Rheological properties were evaluated by a rotary rheometer (Mars II, Haake, Germany).

The protein content in the microalgae samples was estimated using a modified micro Biuret method (paper 3) with some modification.

The amino acid composition was analysed by LC-MS, using EZ: faast^{TM} amino acid analysis kit (Phenomenex Inc. CA, USA).

Lipids were extracted with a mixture of chloroform, methanol, and water, as described by Bligh and Dyer (1959).

Fatty acid profile was analysed by gas liquid chromatography according to the Method described in paper 2.

Analysis of tocopherols and tocotrienols was done by using LC-FLD (Agilent Corporation, Massachusetts, USA), using a mixture of isopropanol and heptane (0.5: 99.5) as the mobile phase (Firestone 2009).

Pigments extraction and analysis were done by the method described in paper 1.

Measurement of moisture was done by an AD 4714A moisture analyzer (A&D Company, Tokyo, Japan). Salt content was measured by a portable salt meter (RHS-10ATC).

The analysis of volatiles was done by a dynamic headspace procedure using the Tenax tube. The separation, identification and quantification was done by GC-MS equipped with MSD Chemstation F.01.03.2357 and Library: Wiley 138K as in Paper 6.

Heat treatment experiments were done in borosilicate glass tubes(25-250 mL), in a temperature controlled water bath. Samples were cooled immediately after heating in a cold water container.

5.2.2 Processing Experiments

Experiments were done in both laboratory and large scales.

5.2.2.1. Harvest (Silicon carbide membrane microfiltration)

Harvest of microalgae biomass was done in batch mode by microfiktration by silicon carbide membranes (SiC) from Liqtech A/S, Denmark. A pilot scale filtration unit (**Figure5. 1a**) was designed and constructed for laboratory scale trials. Tubular SiC membranes (Ø25x 305 mm, Ø 3 mm channel) with different pore sizes (0.04, 0.1 and 1μm) were evaluated. For large scale experiments, a prototype unit for cross-flow filtration was kindly made available for harvesting experiments by LiqTech International (Denmark). The unit (**Figure 5.1b**) was fully automated and also included options for setting back-pulse, back-flush and CIP (cleaning in place). The high flux Co Mem® asymmetric 0.04 μ silicon carbide (SiC) membrane from LiqTech was used. The unit may be operated in both cross flow dead-ended modes with fast forward flush. The Co Mem® elements are designed for cross flow operation. The unit includes seven 1178 mm membrane, which were installed in parallel in a stainless steel housing representing a membrane area of 2.38 m², allowing a feed stream flow of 11 m²/h at linear flow of 2 m/s. In batch mode filtration and for both laboratory and large scale units (**Figure 5.2**), harvested algae recycle back to the feed tank, so the concentration of the feed increases to a constant level (final concentration).

The capacity of laboratory scale microfiltration unit was 20 L/h, and the unit was used for filtration test of different microalgae species. 10-20 L of growth medium (feed) was utilised for each species. For marine species, salt content was also evaluated in both permeate and harvested phases.

(a) **(b)**

Figure 5.1. Cross-flow microfiltration units ;(a) laboratory scale (20-50 L/h) and; (b) Industrial scale (2000 L/h) from Liqtech international A/S (Copenhagen, Denmark)

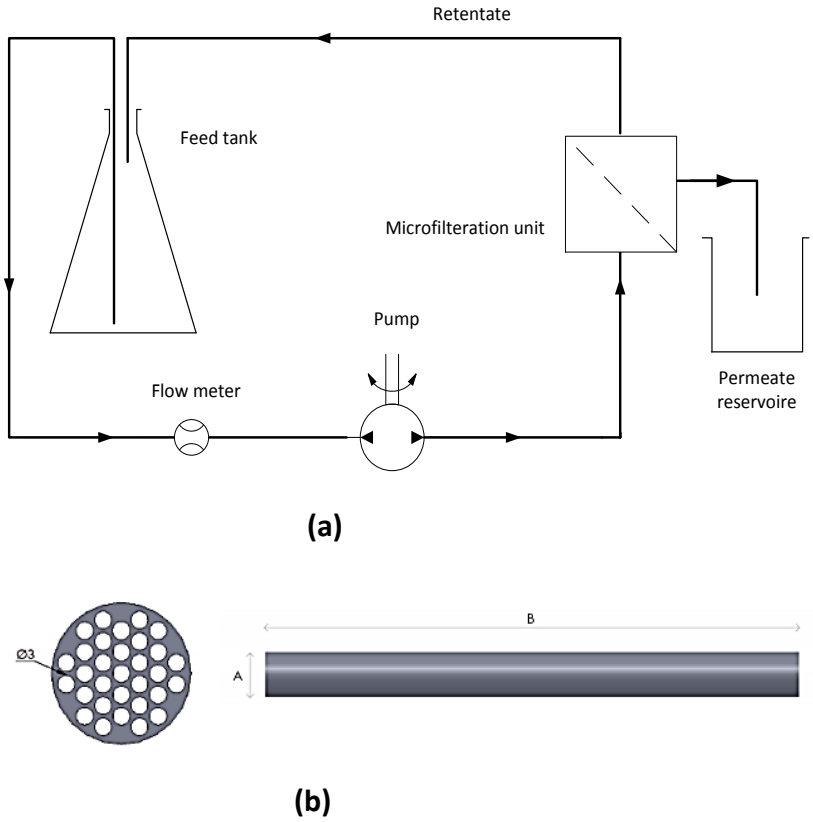


Figure 5.2. (**a**) Schematic of the batch mode microfiltration process and (**b**), dimensions of a single tubular cross flow silicon carbide (SiC) membrane; A=025 mm, B=305 mm

Calculation of filtration rate and concentration factor was done by the following formulae (Cross, 2002; EPA, 2005) :

Flux:

$$J = \frac{Q_p}{A_m} \quad (1)$$

Where:
J = flux, L/hr/m^2; Qp = filtrate flow rate through membrane, L/h; Am = surface area of membrane, m2

Average Flux;

$$J_{avg} = J_0 - 0.33(J_0 - J_f) \quad (2)$$

Where:
J_{avg}= average flux rate; J_0 =initial flux; J_f = final flux

Filtration area:

$$A = \frac{V_0 - V_F}{T \cdot J_{avg}} \quad (3)$$

Where:

A=membrane area; V_0= initial volume; V_F=final volume; T= filtration time; J_{avg}= average flux rate.

Concentration ratio $\overline{CF}$

Concentration ratio was calculated by following equation;

$$\overline{CF} = \frac{C_F}{C_0} \quad (4)$$

Where:

C_F = final concentration of a given solute; C_0 = initial concentration of the solute.

5.2.2.2. Up concentration

A bench scale centrifuge was used for up-concentration of harvested microalgae. Different relative centrifugal forces (RCF) were investigated as; 1000- 15,000 g at various time and temperature setups. RCF was calculated from the following formula:

$RCF = 1.118 \times 10^{-5} \cdot r \cdot (rpm)^2$ (5)

Where; *r*=radius of centrifuge head and *rpm*=rotation speed as round per minutes. Biomass recovery calculated as:Dm_0

$$\text{Biomass recovery (\%)} = \frac{Dm_0 - Dm_p}{Dm_0} \times 100 \quad (6)$$

Where Dm_0 = The initial dry matter (g/L) before centrifugation, and Dm_p= Dry matter in the permeate.

For each trial, 500 ml of the sample was used. Semi-large scale up concentration trials was made by a prototype Skvader decanter centrifuge (**Figure 5.3**) from Alfa Laval Corporate AB (Lund, Sweden).

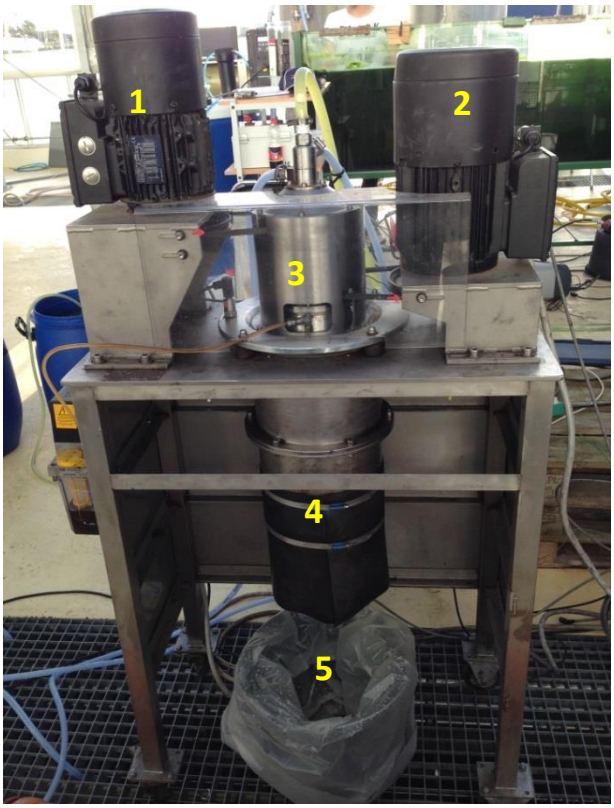


Figure 5. 3. Bowl decanter centrifuge from **Alfa Laval** A/B; 1 and 2- Motors; 3- Gearbox; 4- Separator; 5- collector

5.2.2.3. Drying experiments

A prototype- novel swirl dryer system (**Figures 5.4, and 5.5**) was designed and constructed and was used for drying trials on a *Nannochloropsis salina* paste (22% DM), which was prepared in large scale experiments.

Figure 5.4. Schematic of prototype dryer; 1-Feeding unit: (5 kg paste/h); 2- Air supply belt; 3-Heating unit; 4- Drying tube; 5- Blower; 6- Rotor; 7-Control units.

Microalgae paste was introduced to the drying unit by a peristaltic pump, at a low flow rate (5 ml/min) and directed to the first scraping paddle; maximum inlet air temperature is 120℃. Drying time depends on the species, and biomass composition and particular conditions such as the proportion of free water to bound water is normally less than 5 seconds. Incoming airflow provides the heat requirements. Scraping-fluidizing paddles prevent the material to adhere to the walls, distribute the incoming feed to smaller particles and provide the fluidising spiral flow which moves

the particles to the outlet. Particles move upward via a spiral pattern close to the walls. Bigger particles move back to the scraping section.

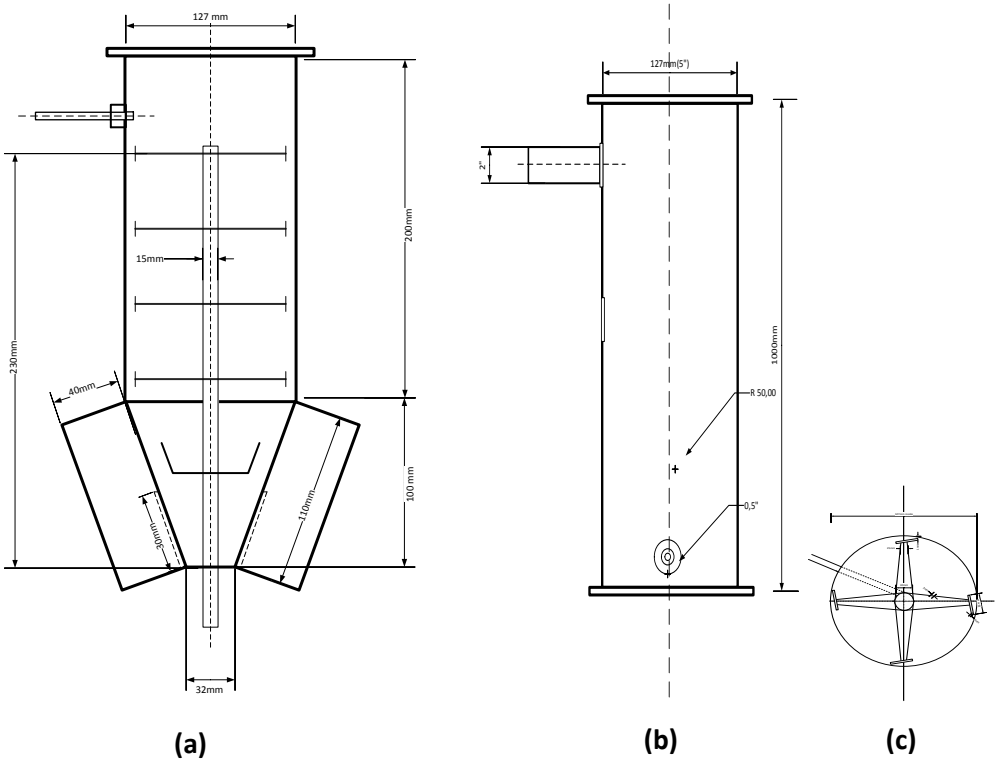
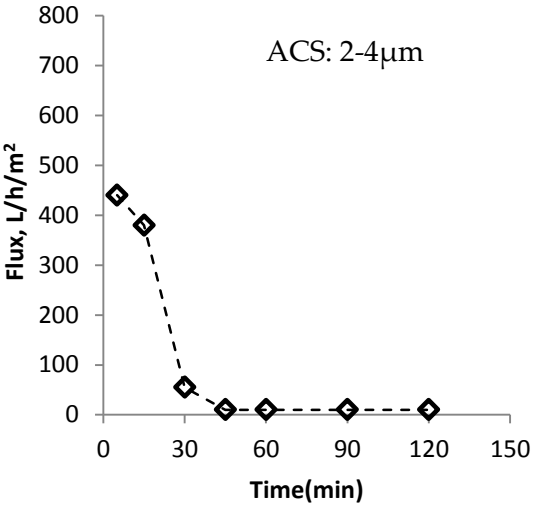


Figure 5.5. Schematic drawing of the prototype dryer; (a) main part including the swirl paddles;(b) drying tube and (c) paddles arrangement cross section.

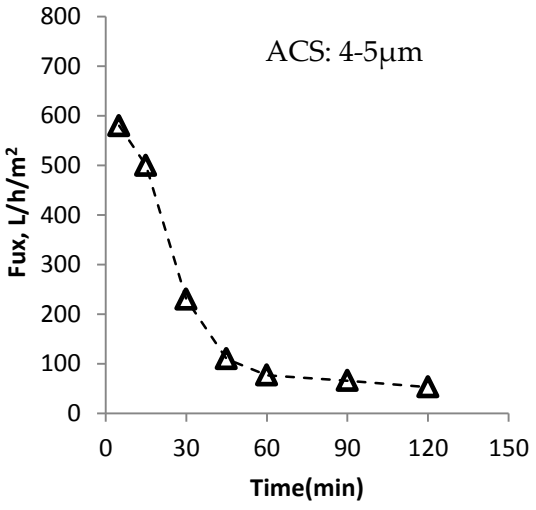
5.3. Results and discussions

5.3.1. Harvest by microfiltration

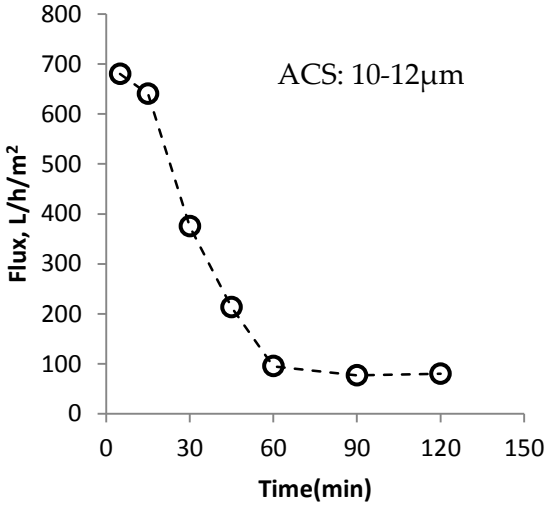
Results of harvest experiments for eight different microalgae obtained by a SIC ceramic membrane (300 mm x 1.0 μm ceramic membrane at constant pressure 1.2±0.2 bar, 20±3℃), are shown in **Figures 5.6 and 5.7.** All of the species showed the same flux pattern. However, flux declined as a result of gradual membrane fouling during the time. Under constant operating conditions, flux declined for all species and then reached an apparent steady state after 120 min. On the other hand, the performance of the filtration was not the same for all species and could apparently be attributed to the microalga cell size and shape. For *Monodopsis subterranea,* decline rate was very fast and caused a membrane fouling in 120 min, while in opposite to these microalgae, *Phaodactylum tricornutom* (**Figure 5.6**), the harvest rate was very fast and reached the constant flux after 60 min, still with high flux.



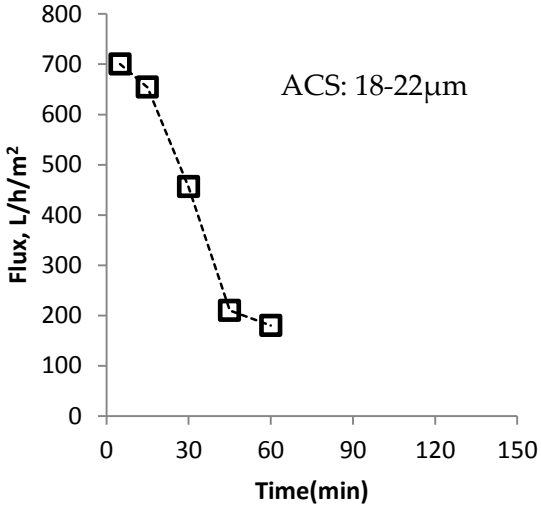
(a)Monodopsis subterranea



(b)Nannochloropsis salina

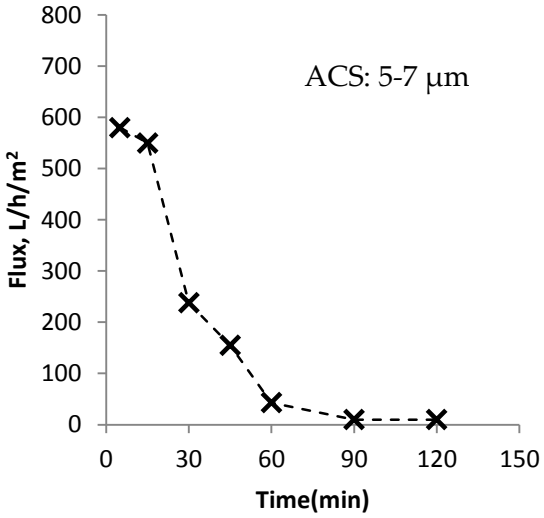


(c)Dunaliella salina

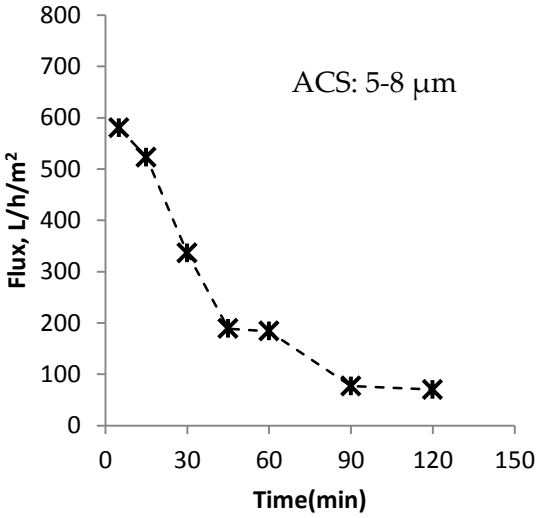


(d)Phaeodactylum tricornutum

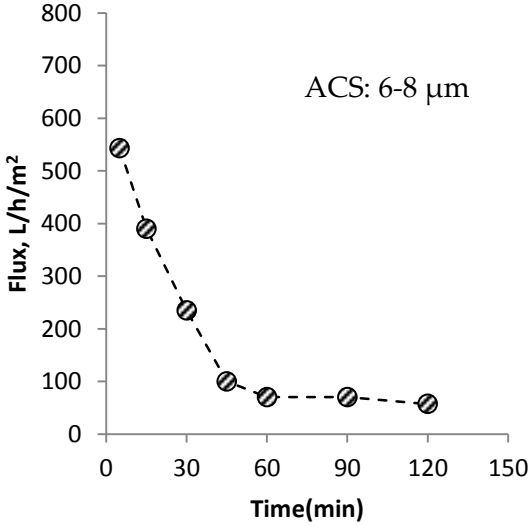
Figure 5.6 Harvest experiments of four microalgae species, (a) *Monodopsis subterranea;(b) Nannochloropsis salina;(c) Dunaliella salina;(d) Phaeodactylum tricornutum* by a 300 mm x 1.0 μm ceramic membrane at constant pressure 1.0±0.2 bar, 20±3℃. ACS= Average Cell Size.



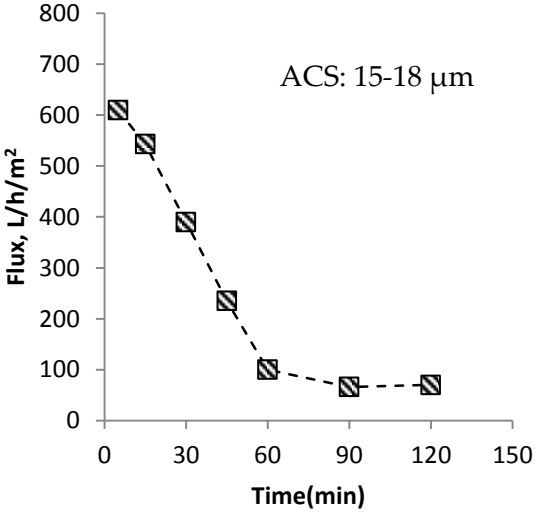
(a)Chlorella vulgaris



(b)Chlorella sorokiniana



(c)Chlorella pyrenoidosa



(d)Desmodesmus sp.

Figure 5.7. Harvest experiments of four microalgae species, (a) *Chlorella vulgaris ;(b) Chlorella sorokiniana;(c) Chlorella pyrenoidosa;(d) Desmodesmus* sp. by a 300 mm x 1.0 μm ceramic embrane at constant pressure 1.0±0.2 bar, 20±3℃.

Harvest of *Dunaniella salina* with an average cell size of 10-12 μm started with higher flux, compared to *Nannochloropsis salina* and *Monodupsis subterranea*, and reached a constant rate after 120 minutes. *Chlorella sorokiniana*, *Chlorella vulgaris*, *Chlorella pyrenoidosa* and *Desmodesmus* sp. harvest also showed nearly the same pattern (**Figure 5.7**), despite the fact that average cell size (ACS) for *Desmodesmus* sp. was apparently higher than the *Chlorella* species, so other factors which influence the harvest performance of the microalgae shall also be considered.

Cell wall toughness and cell shape are other important factors which may contribute to the harvest performance of microalgae species. For microalgae with rigid cell wall such as *Phaeodactyllum* sp., the effect of filtration shear on cell rupture is not high, especially when the average pore size of the membrane is much lower than average microalgae cell size, so the filtration is being done very fast and efficient. When the average cell size is close to the membrane average pore size, then some of the microalgae can be squeezed to the pores with the same size which results in a cell rupture. *Dunaniella salina* does not have a tough cell wall (Baroda et al., 2014), but due to a comparatively big cell size, the flux is not influenced at the first 120 minutes of filtration. In microalgae with damaged or thin elastic plasma membrane, this phenomenon causes a decline in flux. Debris from microalgae damaged cells gradually fouled the membrane which reduced the flux. On the other hand, the cell particle also increased the fluid viscosity leading to non-Newtonian viscoelastic behaviour which further reduced the flux (Mc Millan et al., 2013). As shown in **Figure 5.6.**, average cell size for both *Monodopsis* sp. and *Nannochloropsis* sp. was similar, but the filtration performance was higher in *Nannochloropsis* sp., which can be justified with the difference in cell wall toughness.

The concentration ratio and average flux highly varied between different microalgae species, as shown in **Figure 5.8.** The highest concentration ratio was achieved for *Phaeodactylum tricornutum* and the lowest for *Monodopsis subterreana*. Both concentration ratio and average flux correlate well with the average cell size (R^2= 73.9 and 76.4%, respectively), and also with each other (R^2= 75.0 %). In all of the experiments excluding the *Monodopsis subterranea*; the recovery efficiencies were higher or equal to 98%. These findings represent the ceramic membrane microfiltration as an efficient, but species depended harvest method.

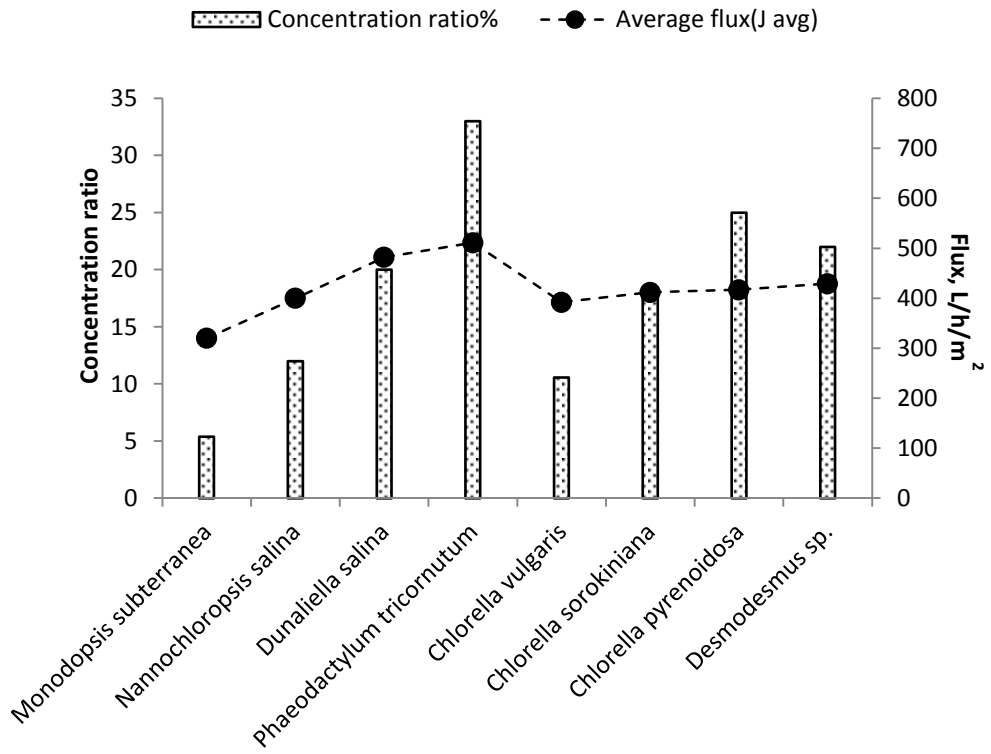


Figure 5.8 Concentration ratio ($\overline{\boldsymbol{CF}}$) and average flux in eight microalgae harvested by a 300 mm x 1.0 μm ceramic membrane at constant pressure 1.0±0.2 bar, 20±3℃.

5.3.1.1 The effect of membrane pore size

In order to evaluate the effect of membrane pore size on harvest performance, a culture of *Nannochloropsis salina* was harvested by 300 mm ceramic membranes with different pore sizes as 0.4, 0.1 and 1.0 μm. Harvest experiments were done at constant pressure 1.3±0.2 bar and temperature 20±3℃. Harvest by 1.0 μm started at higher fluxes (**Figure 5. 9**) but declined to a low flux after 120 minutes. The flux for membranes with smaller pore sizes reached the steady state after 60 minutes, with no difference between 0.04 and 0.1μm membranes. Due to the higher pressure drop, energy consumption increases when the pore size decreases, so a membrane with 0.1 μm pore size would be a better candidate for the harvest of microalgae with the small size range (2-4 μm).

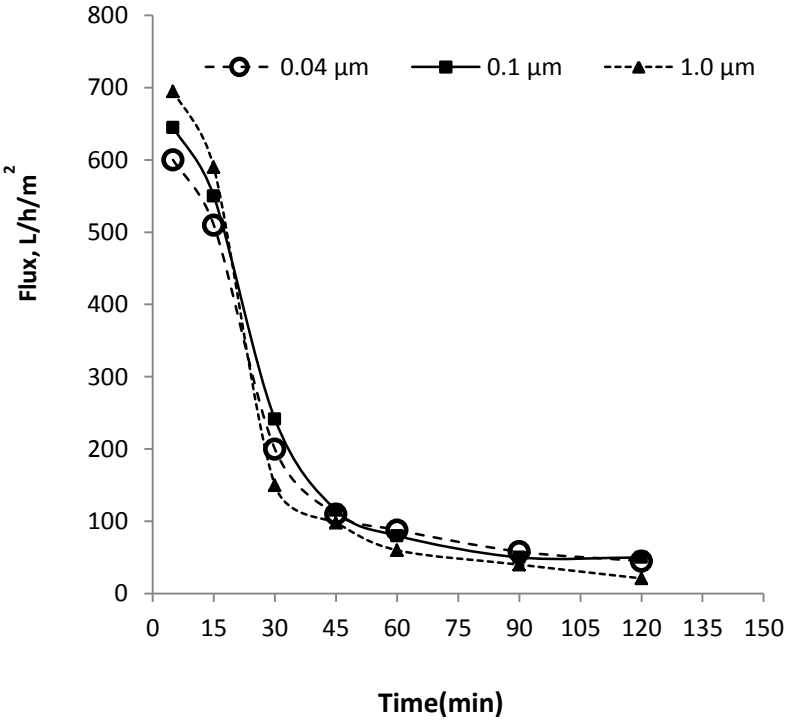


Figure 5.9 Harvest performance(flux) for *Nannochloropsis salina* by 300 mm ceramic membranes with different pore sizes as 0.04, 0.1 and 1.0 µm. Harvest experiments were done at constant pressure: 1.3±0.2 bar and temperature: 20±3℃.

5.3.1.2 Effect of the temperature

Effect of the temperature on the flux for a *Nannochloropsis salina* culture was evaluated (**Figure 5.10**). Harvest performance (flux) started at higher rates at higher temperatures, but declined faster for the 30±5℃ experiment, compared to lower temperatures. This finding can be justified with the fact that microalgae cell wall is more rigid at low temperature (McMillan et al., 2013). On the other hand, the viscosity of fluids increases when the temperature decreases which negatively affect the filtration performance, so filtration in a range of 15-20℃ will be optimum for the harvest of intact, non-ruptured microalgae with the rigid cell wall. However, the temperature control might not always be feasibly possible, e.g. in warm climates.

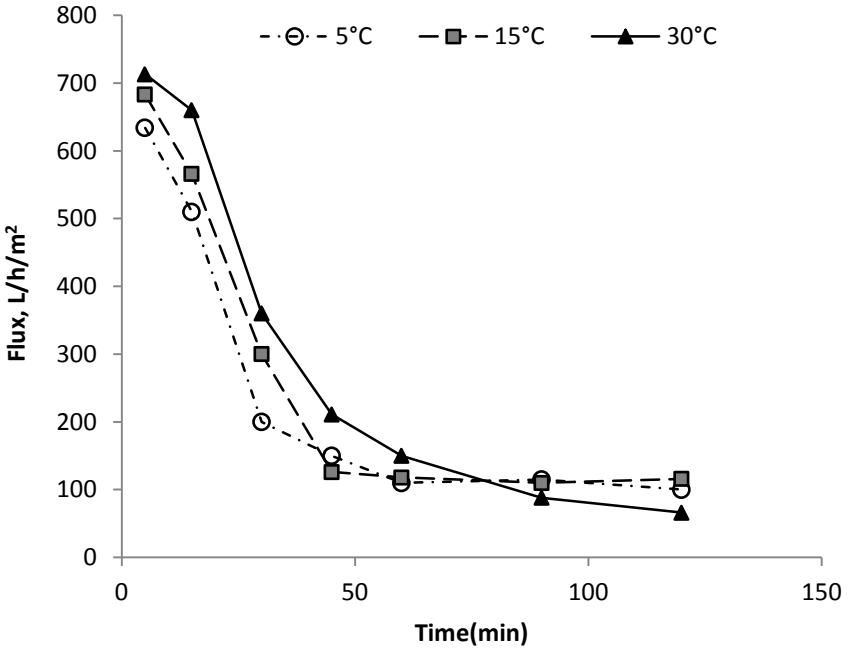


Figure 5.10 Harvest performance (flux) for *Nannochloropsis salina* by 300 mmx0.1 μm ceramic membranes at different temperatures. Harvest experiments were done at constant pressure 1.3±0.2 bar, and temperatures 5±2℃, 15±3℃, and 30±5℃. Temperature of growth media during the harvest was measured.

5.3.1.3 Effect of pressure

Effect of transmembrane pressure at the harvest performance of two culture of microalgae was evaluated (**Figure 5.11 a, and b**). Harvest experiments were done at constant temperature 17±2℃, and different pressures 1±0.1, and 2±0.4 bar. Pressure was measured and controlled at the filter hose outlet stream. The initial concentrations of dry matter were 0.14 and 0.88 g L^{-1} for *Nannochloropsis salina*, and *Chlorella vulgaris*, respectively. In theory, flux increases when the transmembrane pressure increases. However, in a biological fluid like microalgae culture, there are many different factors which can affect the flux during the time and change the filtration efficiency at various pressures. The so-called transmembrane pressure for the SiC ceramic membrane determined as less than 3 bars in preliminary studies (data are not shown here). Higher pressures showed an adverse effect on the long-term harvest efficiency but changed the filtration behaviour of the filter, especially for low pore size membranes, e.g. 0.04 - 0.1 μm membranes (Figure 11a and b). As shown in **Figure 5.11a,** filtration of *Nannochloropsis salina* at 2 bar pressure increased the flux during the harvest for 120 min. Final concentration ratio($\overline{CF}$) was also apparently higher (24.3 times) when compared to the sample harvested at a lower pressure (18 times). For the *Chlorella vulgaris* sample, surprisingly no significant

differences were observed in the harvest efficiency. Concentration ratios were measured as 10.0±0.2 and 10.2±0.1 times for the 1 and 2 bar experiments, respectively. Further investigation revealed that the formation of a pressure resistant filter cake reduced the flux and concentration ratio in this species, which could explain the low efficient harvest results. So while the higher pressure overcame the flow decline induced by the cake layer on the membrane, but the concentration ratio was not increased. It has previously been reported (Babel and Takizawa, 2010) that compounds such as exopolysaccharides, proteins, lipids or humic substances which may release from microalgae due to high shear stress cause severe membrane fouling. Results of this experiment offer a maximum concentration ratio which depends on the processing hydrodynamic condition as well as the culture properties such as initial dry matter concentration, cell size and type, and other compounds in the growth medium, which prevent further concentration after an individual point. The harvest would not be efficient to continue after reaching the maximum concentration level. Otherwise, it increases the processing expenses for both energy consumption and cleaning of the membrane.

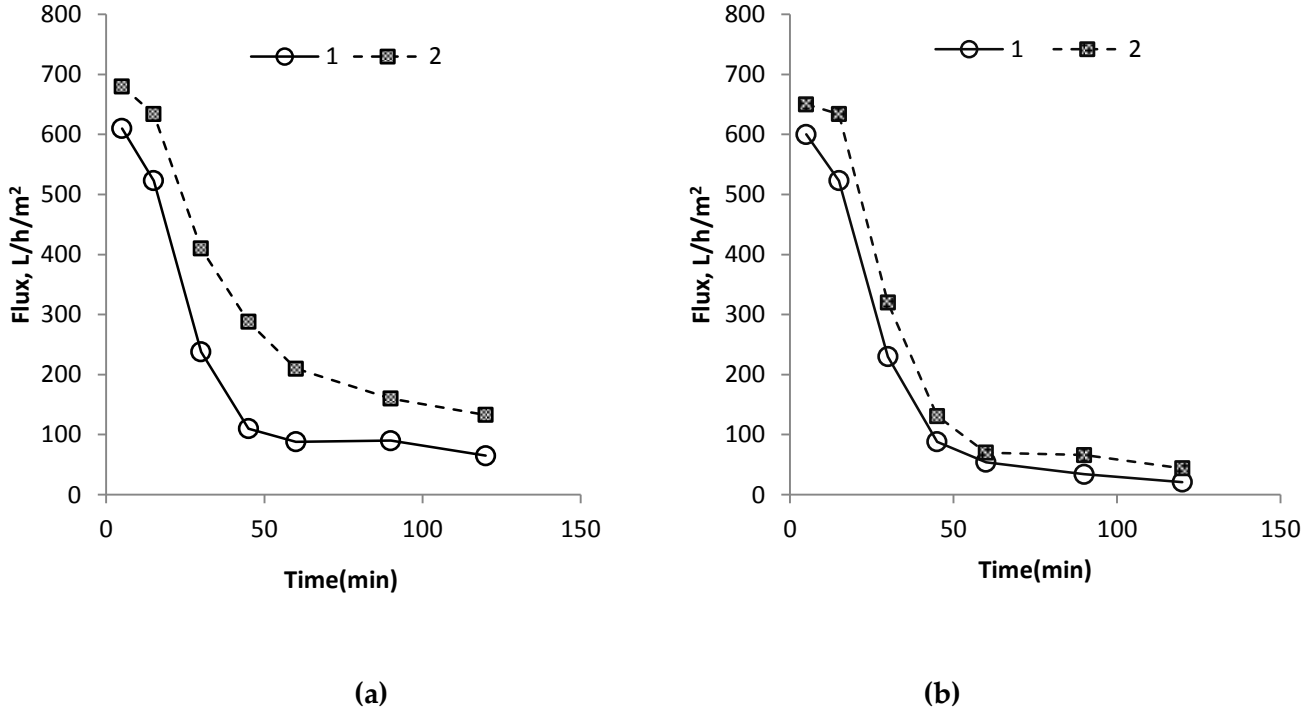


Figure 5.11. Harvest performance (flux) for *Nannochloropsis salina* (***a***) and *Chlorella vulgaris* (***b***) by 300 mmx0.1 μm ceramic membranes at different pressures. Harvest experiments were done at constant temperature 17±2℃,, and different pressures 1±0.1, and 2±.2 bar. Pressure measuares and controled at the filter hose outlet stream. The initial concentration of dry matter for two species under experiment were 0.14 and 0.88 g L^{-1} for *Nannochloropsis salina*, and *Chlorella vulgaris*, respectively.

Ahmad et al.(2012) used a cellulose acetate membrane for the harvest of *Chlorella vulgaris* and reported that the permeate flux increases with increased transmembrane pressure. The study suggested that the high pressure increases the resistance of the membranes to mass transfer; and it prevents the microalgae from settling on the membrane surface. Fouling of the membranes is one of the primary limitations that can reduce the performance of harvest during the microfiltration (Slater et al., 2015). The membrane fouling in addition to hydrodynamic factors also depends on biological parameters such as microalgae concentration, cell size, cell wall strength, and the concentration and type of other biological compounds/ organisms which may exist in the growth media. It has previousely been shown that by increasing the pressure, the formation of cake occurs faster and causes higher cake resistance (Babel and Takizawa, 2010). In many cases, the culture includes symbiosis organisms (bacteria) or even zooplankton which feed on microalgae and may also generate the above mention extraneous biopolymers and cause membrane fouling.

Javadi et al. (2014) investigated the effects of operating conditions such as transmembrane pressure, flow rate and optical density of feed suspension on the performance of microfiltration for the harvest of *Chlorella* sp. The study suggested gas sparging technique as an efficient method especially in low concentration microalgae microfiltration, which could enhance the efficiency of harvest process, by preventing the membrane fouling. In another study (Nura et al., 2014), regardless of the type of microalgae and hydrodynamic conditions, dynamic filtration was suggested as the best technology over a conventional method. The study used a shear-enhanced vibratory microfiltration process for the harvest of microalgae which improved the filtration by prevention of fouling. As a conclusion methods such as back pressure or sparging are more efficient than increasing the transmembrane pressure.

5.3.1.4 Remaining salt in the retentate

In order to investigate the permeability of SiC membrane to the salt, a culture of marine microalgae *Nannochloropsis salina* was harvested by a 300 mmx0.1 μm SiC ceramic membrane (**Figure 5.12).** During the microfiltration of marine microalgae, the salt content is being washed out into the permeate phase. This phenomenon is a significant advantage of the microfiltration harvest technique, compared to methods such as centrifugation which requires additional washing procedure to remove the salt from the biomass. On the other hand, the permeability of salt and salt complexes is not the same for different membrane matrices. In our study, remaining salt content correlates well with the concentration ratio. In other words, higher concentration ratio equals less salt content in the retentate.

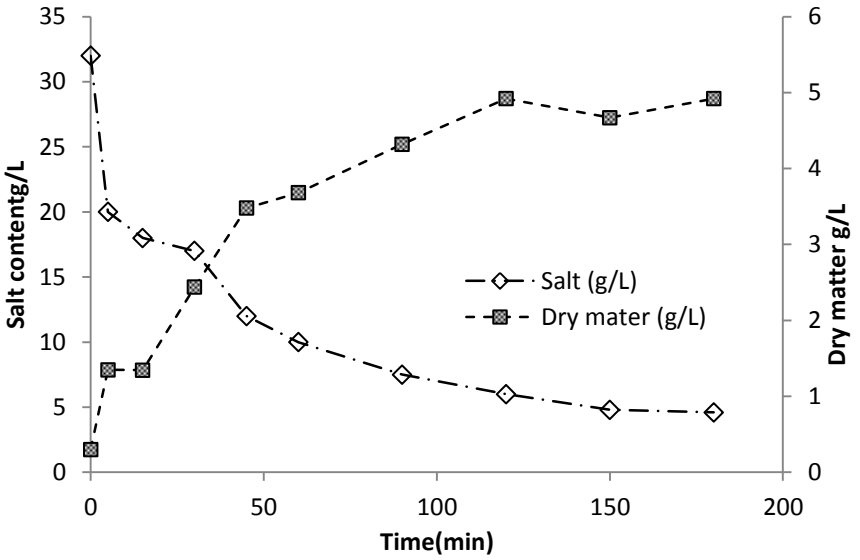


Figure 5.12 Effect of concentration ratio on the salt content in marine *Nannochloropsis salina* culture. Microfiltration was performed by 300 mmx0.1 μm SiC ceramic membrane at constant temperature 17±2℃, and pressure of 1. 5 ±0.25 bar. The initial dry matter of the culture was 0.3 g L^{-1} and the initial salt concentration was 32 g L^{-1}.

5.3.1.5 Large scale trials:

Harvest trials were performed on *Chlorella sorokiniana* in 4000 liter continuous mode. Starting concentration ranged between 0.90 ± 0.1 g/L. A final concentration of up to 70 g/L could be achieved, but in order to optimize energy costs, reduce share stress and fouling, an optimum of 20 g/L was achieved. Further concentration is possible, but resulted in membrane fouling, and higher shear stress due to the increased pressure. Unfortunately test of 0.1 μm membrane was not possible at this point in time. In general trials resulted in a concentration ratio of 20-40 times and water removal of >90% (mostly extracellular water). Tests were run at a flux range of 85-150 L/h/m^2 (**Figure 5.13**). As shown it has shown, membrane fouling did occur during test runs. However, fouling could be reduced by using 0.1 μm membrane as shown in lab trials.

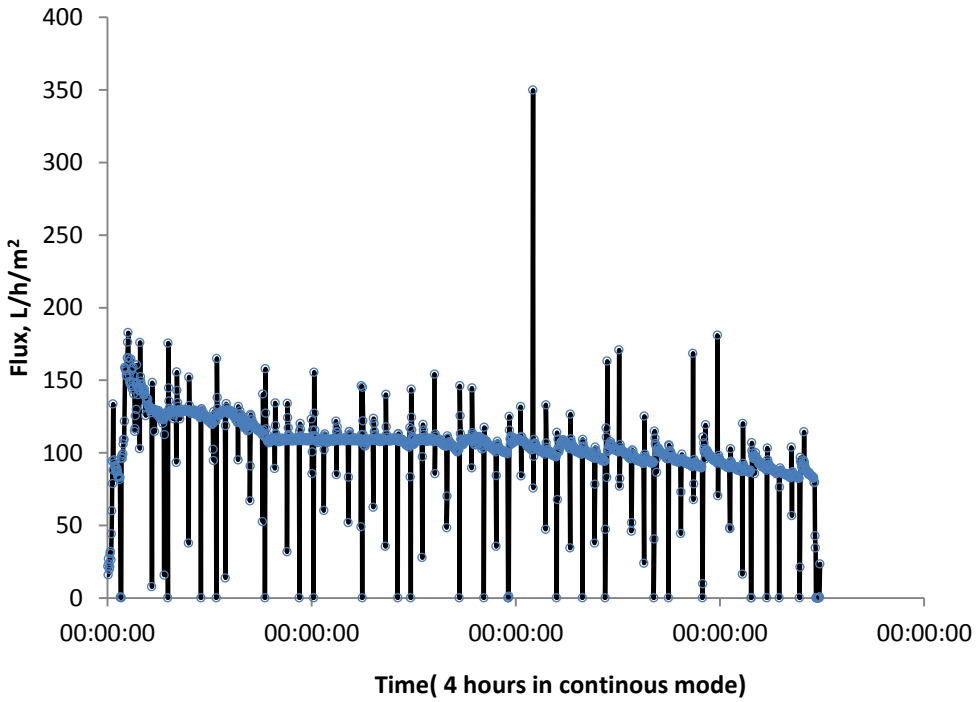


Figure 5.13 Large scale harvest experiments of *Chlorella vulgaris ;(b) Chlorella sorokiniana.* Unit consisted of seven 1178 mm x 25 mm membranse which were installed in parallel in a stainless steel housing. The total membrane area was 2.38 m², allowing a feed stream flow of 11 m²/h at linear flow of 2 m/s. Flux values represent the data in constant phase(after first 30 minutes of running).

5.3.2 Centrifugation for the up concentration of harvested microalgae

During the cultivation and downstream processing, microalgae cells are being affected by hydrodynamic forces from mechanical processes such as pumping, mixing or centrifugation (Milledge&Heaven 2011; Xu et al.,2015). These external forces could cause cell wall rupture when they are big enough. Microalgae cell wall strength is not the same, so some species are more sensitive to such external stresses. Smaller forces cause cell death without membrane fracture. This phenomenon may happen in flat panel photobioreactors or during the mechanical displacement (Sung et al., 2015). Molina Grima et al. (2010) suggested that when the size of g-force induced micro-eddies are equal or smaller than the size of microalgae cell, then the mechanical forces applied to the cell wall is higher than its strength and as a result, cell rupture occurs. Eddy is defined as the reverse force created by swirling of fluids when the fluid flows past an obstacle. Disruption of the membrane may occur as a result of localised velocity gradients caused by one or more micro eddies (Milledge&Heaven, 2011).

The size of micro-eddies was estimated as 7μm for an individual disc stack centrifuge (Milledge&Heaven, 2011), which falls in the cell size range for many microalgae species. On the other hand, the strength of cell wall highly varies amongst microalgae species, so that the cell size can not justify the effect of external hydrodynamic forces just by itself. Cell rupture has both active and adverse consequences, depending on the aim of the process. When the microalgae biomass is supposed to be used as a fish feed ingredient, then the quality of nutrient has a great importance. To optimise the centrifugation process, different *g*-forces (1000-15,000) were applied, and the effect of centrifugal force on the physiochemical properties of the biomass were evaluated. All microalgae cultures were harvested previously by a SiC ceramic membrane (300mm x 0.1μm), at room temperature and 1.2±0.2 bar pressure.

5.3.2.1 Biomass recovery

Biomass recovery pattern in different relative centrifugal forces (RCF) as g for several microalgae species including N*annochloropsis salina, Chlorella vulgaris, Chlorella sorokiniana,* and *Desmodesmus* sp., was evaluated by a bench scale batch centrifuge (Sorvall RC 6 Plus from Thermo scientific, Waltham, MA, USA). Centrifugation was done at constant temperature 20 ±1℃, and for 10 minutes, in 500 ml polyethene centrifuge tubes. Yield of precipitation (%) depends on several factors such as cell size, growth stage, the percentage of damaged/dead cells and the initial concentration of the culture. As shown in **Figure 5.14**, for RCF values more than 6000 g, biomass recoveries were estimated to be higher than 99.2 %. Deviations in the biomass recovery at lower RCF was high. The recovery for small size and damaged microalgae cells was lower than the others. When RCF values greater than 6000 g were applied, regardless of the parameters mentioned above, for sound microalge cells, nearly all of the biomass was recovered. Physical and chemical properties of the up concentrated biomass are different. According to this model, the ideal RCF for the recovery of the microalgae falls in the range of 6000 g, which compared to the higher values, require less energy, while factors such as dry matter in the biomass and physiochemical properties shall also be considered.

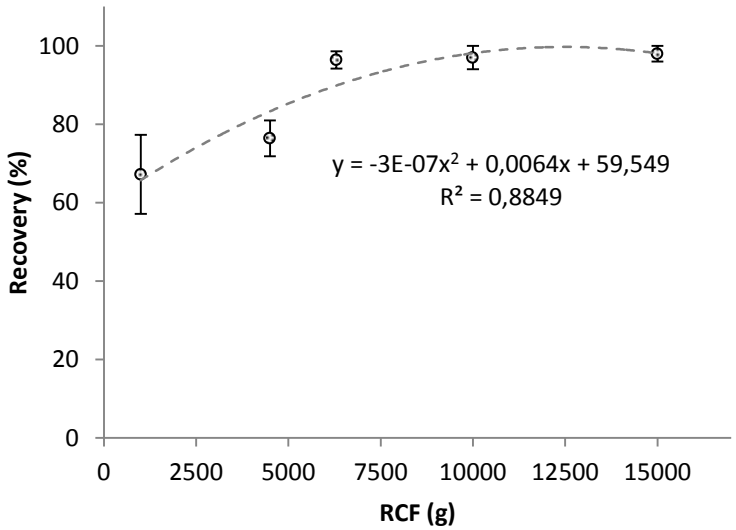


Figure 5.14. Microalgal biomass recovery pattern in different RCF(g). Data was collected from up-concentration trials of several microalgae species (at different growth stages) including *Nannochloropsis salina, Chlorella vulgaris, Chlorella sorokiniana,* and *Desmodesmus* sp.*;* Centrifugation was done at constant temperature 20 ±1℃, and for 10 minutes*(n=3).*Bars are showing the standard deviations for each RCF.

5.3.2.2 Variations in lipid and protein contents

Effects of centrifugation at different RCF's were evaluated on *Nannochloropsis salina* and *Chlorella sorokiniana*. The content of protein increased in centrifuged samples, especially at higher RCF values, compared to the harvested sample. For *Chlorella sorokiniana*, variations in protein content were more apparent than *Nannochloropsis salina*. For the sample centrifuged at 15000 g protein content measured as 34±1.13 %dw, which compared to the harvested sample (32±0.96 %dw) was significantly higher (**Figure 15 a** and **b**). On the other hand, lipid contents decreased when the RCF increased for both species. Variations in lipid content were also higher for *Chlorella sorokiniana.* These results could be justified with the fact that average cell size for this microalga (5-9 μm), compared to the *Nannochloropsis salina* (2-4 μm), was bigger, so the degree of rupture caused by centrifugation (such as micro eddies) was higher. Due to the rupture, some of the lipids (mostly membrane lipids) were liberated to the water phase, while the extractability of the proteins are increased for the same reason.

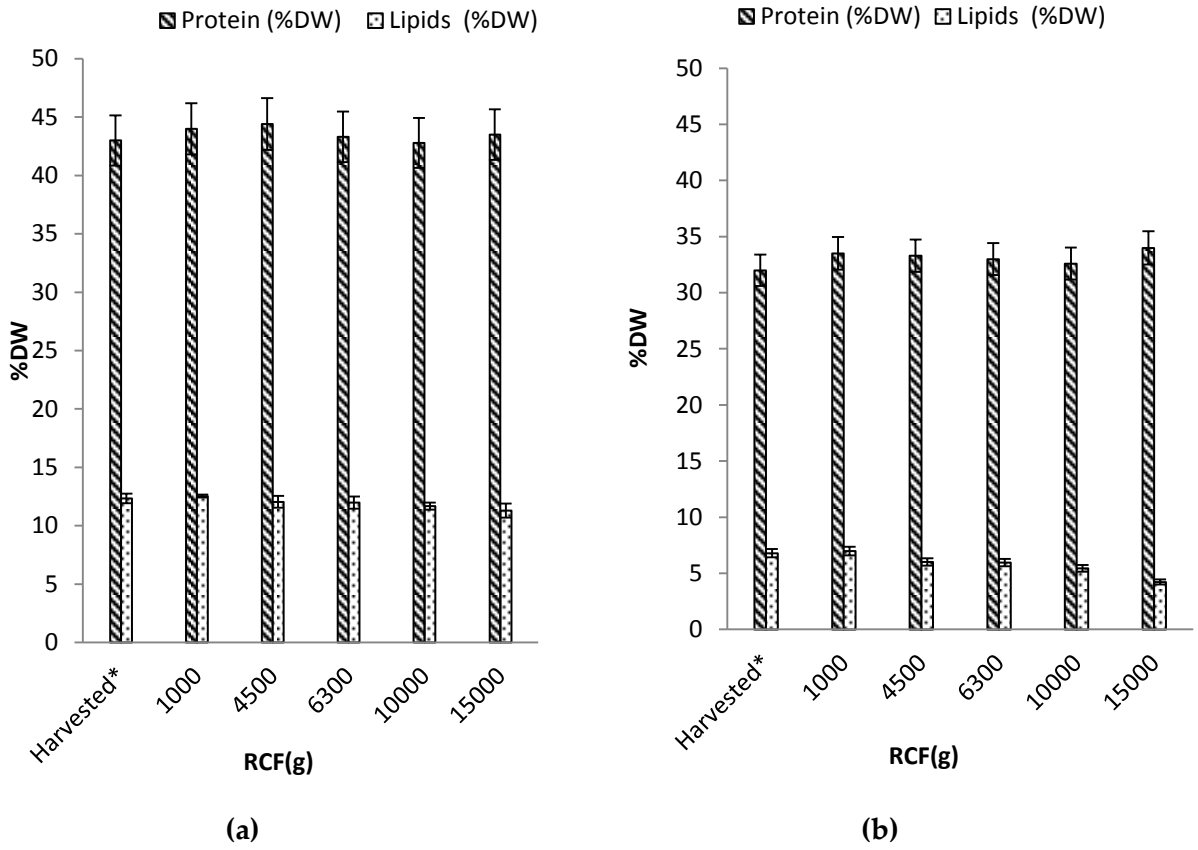


Figure 5.15. Lipid and protein contents(%DW) variations in;(a) *Nannochloropsis salina* and;(b) *Chlorella sorokiniana* paste prepared in different RCF's(1000-15,000).* Harvested by microfiltration. Bars show the standard deviations(n=2).

5.3.2.3 Variations in fatty acid composition

Variations in the fatty acid composition in a culture of *Nannochloropsis salina* wit high content of polyunsaturated fatty acids were investigated. As shown in **Table 5.1,** when the RCF value increased, the contents of polyunsaturated fatty acids decreased. In microalgae, membrane lipids include polyunsaturated fatty acids such as eicosapentaenoic acid (EPA). So at the high RCF values, cell wall rupture results in loss of these compounds.

Table 5.1 Fatty acid composition of *Nannochloropsis salina* paste prepared in different RCF.

	RCF(g)					
Fatty acid:	**Harvested***	**1000**	**4500**	**6300**	**10000**	**15000**
14:0	3.23±0.37	3.02±0.07	3.20±0.01	3.22±0.07	3.15±0.05	3.28±0.16
15:0	0.23±0.04	0.29±0.01	0.29±0.01	0.31±0.01	0.30±0.00	0.31±0.01
16:0	17.7±0.04	19.4±0.08	18.9±0.15	20.4±0.17	19.6±0.01	20.0±0.33
16:1 (n-7)	26.3±0.19	28.7±0.15	28.6±0. 4	30.0±0.43	29.2±0.08	31.5±1.61
16:2(n-4)	0.09±0.02	1.11±0.06	1.22±0.03	1.16±0.03	1.13±0.02	1.15±0.02
16:3(n-4)	0.20±0.03	0.19±0.00	0.19±0.00	0.20±0.01	0.19±0.01	0.18±0.01
17:0	0.00	0.31±0.05	0.32±0.02	0.28±0.01	0.27±0.01	0.25±0.01
16:4(n-1)	0.45±0.02	0.09±0.01	0.09±0.01	0.08±0.01	0.09±0.00	0.09±0.00
18:0	0.23±0.00	0.39±0.00	0.31±0.01	0.31±0.02	0.27±0.00	0.26±0.04
18:1 (n-9)	1.69±0.03	1.80±0.02	1.71±0.01	1.77±0.14	1.56±0.02	1.49±0.19
18:1 (n-7)	0.85±0.01	0.94±0.42	0.75±0.17	0.79±0.21	0.43±0.03	0.44±0.09
18:2 (n-6)	0.14±0.00	1.37±0.02	1.50±0.01	1.37±0.01	1.39±0.01	1.36±0.06
18:2(n-4)	0.89±.03	0.90±0.01	1.02±0.00	0.93±0.04	0.97±0.01	0.93±0.06
18:3(n-3)	0.94±0.01	0.38±0.06	0.37±0.06	0.28±0.01	0.26±0.03	0.26±0.01
18:4 (n-3)	0.13±0.01	0.13±0.00	0.14±0.00	0.13±0.00	0.14±0.00	0.14±0.01
20:4 (n-6)	2.64±0.03	0.00	0.00	0.00	0.00	0.00
20:3 (n-6)	0.87±0.01	0.49±0.15	0.55±0.04	0.52±0.06	0.57±0.05	0.52±0.04
20:3(n-3)	0.06±0.01	0.00	0.00	0.00	0.00	0.00
20:4(n-3)	2.55±0.04	2.26±0.16	2.64±0.01	2.39±0.07	2.44±0.01	2.33±0.13
20:5 (n-3)	39.5±0.23	37.9±0.59	38.1±0.33	37.4±0.16	35.8±0.25	35.3±1.33
21:5(n-3	0.09±0.01	0.00	0.00	0.00	0.00	0.00
22:5 (n-3)	0.06±0.00	0.00	0.00	0.00	0.00	0.00
22:6 (n-3)	0.09±0.01	0.19±0.09	0.13±0.04	0.33±0.23	0.16±0.01	0.16±0.04
Total unsaturated	77.6±0.63	76.5±1.69	76.9±0.71	75.41±1.36	76.3±0.51	75.8±3.56
Total n-3	43.42±0.30	40.9±0.91	41.4±0.45	40.5±0.47	40.8±0.30	38.1±1.51

*results are presented as % of total fatty acids(n=2)

5.3.2.4 Pigments

Effects of centrifugation on the pigment composition in two microalgae species, *Nannochloropsis salina* and *Chlorella sorokiniana* were investigated (**Figure 5.16a and b**). For all pigments including chlorophylls, carotenes and xanthophylls, higher concentrations were achieved in the samples which

were centrifuged at higher RCF values. These results can also be attributed to the cell wall rupture which happened at high centrifugation forces. Xu et al. (2015) also showed that hydrodynamic forces caused by the centrifugation did not degrade the sensitive pigments such as xanthophylls. Carotenoids accumulate as droplets in the chloroplasts and recover within the microalgae cell during the centrifugation.

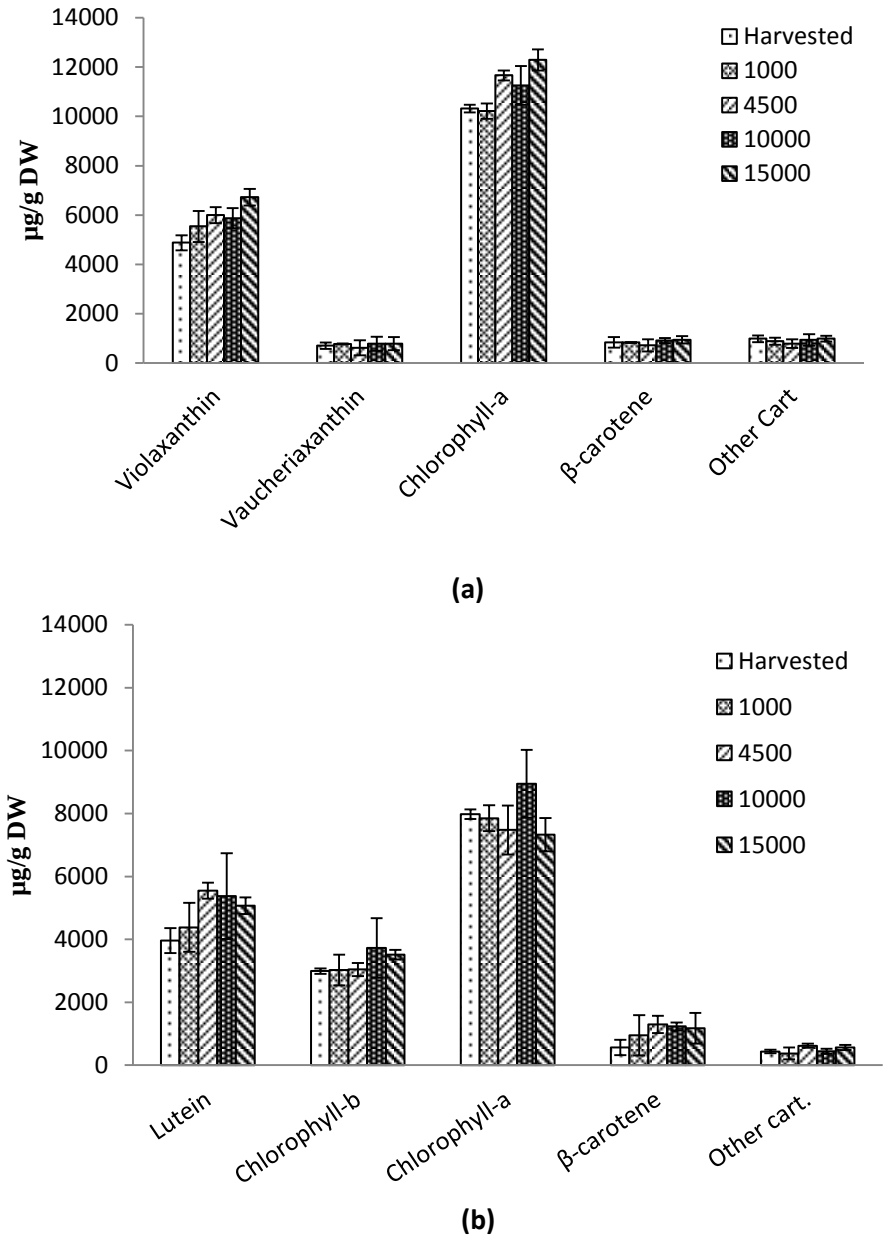


Figure 5.16. Pigment composition(%DW) variations in;(a) *Nannochloropsis salina* and;(b) *Chlorella sorokiniana* paste prepared in different RCF's(1000-15000).).* Harvested by microfiltration. Bars show the standard deviations(n=2).

5.3.2.5 Effects of centrifugation force on leakage and physical properties of resulting paste

In **Figure5.17**, the physical appearances of microalgae paste separated at various RCF's are shown. The pastes were separated from a *Chlorella sorokiniana* culture, which was harvested by microfiltration and then centrifuged at various RCF's 1000-10,000 g. The dry matter concentration of harvested medium was 18.4 g L^{-1}. The presence of an emulsion phase (in pinkish-orange colour) even at lower RCF was surprising. Xu et al. (2015) found that cell rupture occurs at RCF equal or bigger than 5000 g for *Dunaliella salina*, in which cell wall strength is low, so as this species has rigid cell walls, this emulsion layer can be attributed to the leakage of lipid compounds. The formation and amount of this emulsion phase depended on the hydrodynamic forces applied to the microalgae cell during the centrifugation, content and composition of the lipids, cell size, culture age, species and cell wall thickness.

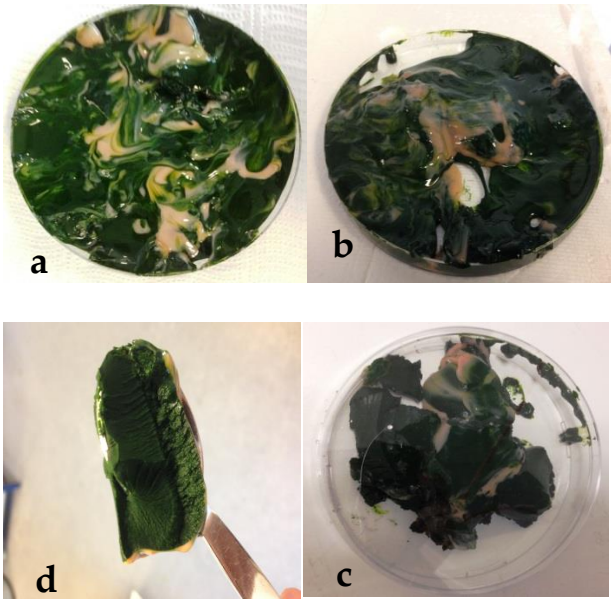


Figure 5.17. The physical appearance of *Chlorella sorokiniana* biomass paste separated at different RCF values;**(a),**1000 g;**(b),** 4500 g; **(c),** 6300 g and **(d),** 10000g. Centrifugation was done at constant temperature 20 ±1℃, and for 10 minutes. Dry matter concentration of harvested medium was 18.4 g L^{-1}.

It has previously been shown that during the centrifugation, various stresses such as a hydrodynamicforce from turbulence, viscous drag, a cell weight increase due to the centrifugation and also hydrostatic pressure from the liquid head are being applied to the microalgae cell (Xu et al. 2015). The combination of these external forces causes rupture or a leakage of the lipids to the water phase. For microalgae with bigger cell size and thinner cell walls, this phenomenon could result in a

loss of 40% of the lipid (Xu et al., 2015). Lipid content (both membrane and intercellular lipids), cell wall strength, amounts of damaged cells because of previous mechanical stresses and cell size are critical factors that could affect the degree of leakage in microalgae. In **Figure 5.18**, the percentage of the leakage (%DW of biomass) for two microalgae species, *Nannochloropsis salina* and *Chlorella*

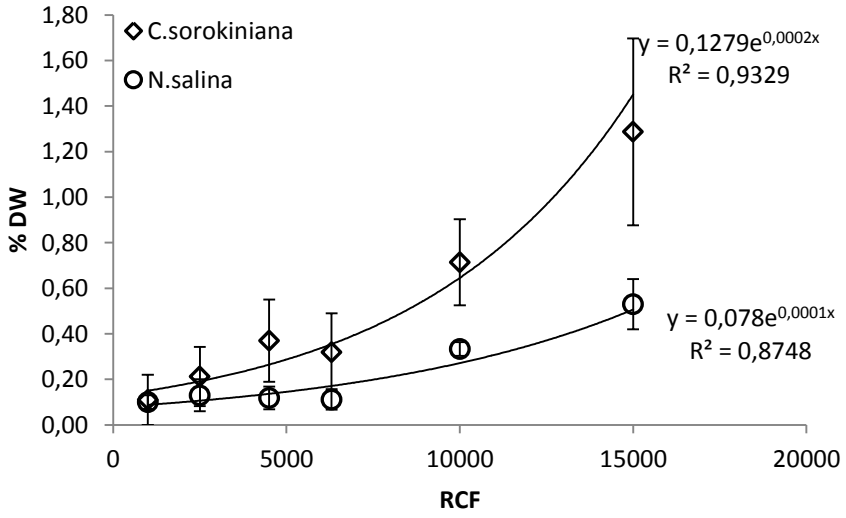


Figure 5.18 The relationship between the percentage of leakage and RCF values (1000-15000 g), for *Chlorella sorokiniana* and *Nannochloropsis salina*. Both cultures were previously harvested by SiC ceramic membrane.Centrifugation was done at constant temperature 20 ±1℃, and for 10 minutes for both species(n=7).

sorokiniana against RCF(g) are plotted. For *Chlorella sorokiniana* with bigger average cell size, the degree of the leakage is much higher, offering the fact that centrifugation of microalgae with bigger cell sizes or thinner cell walls shall be done in lower RCF values. For microalgae with the cell size in the range of 5-8μm, a RCF of 6500 g could be recommended. On the other hand, centrifugation can be considered as a rupture cell method when required. A disc stack centrifuge normally operates at 11,000-15,000 g (Milledge and Heaven, 2011) so rupture and leakage of lipid fraction inevitably occur. In this case separation of the lipids from the water phase requires another centrifuge. The percentages of leaked phase also depends on the degree of cell rupture, contents and composition of the lipids in the membrane and cellular lipids. In **Table 5.2** and **5.3** the fatty acid composition of leaked emulsion from Nannochloropsis salina and *Chlorella sorokiniana* are shown, respectively. To our knowledge the composition of leaked phase from centrifuged microalgae was not studied before. In *Nannochloropsis salina*, fatty acid composition of the leaked phase is similar to the green phase and harvested (non-centrifuged) biomass, offering that the lipid profile of the membrane and intercellular lipids are the same (**Table 5.2**). In contrast, the composition of the fatty acids in the leaked phase separated from *Chlorella sorokiniana* was quite different compared to the non-centrifuged culture and the biomass

phase, and revealed high variations in the fatty acid compositions between the different phases (**Table5.3).**

Table 5.2. Fatty acid composition of the leaked phase from *Nannochloropsis salina* paste which was separated by centrifugation at 4500 g.

Fatty acid	Harvested	biomass phase	leaked phase
14:0	2.38±0.05	2.15±0.08	2.47±0.01
15:0	0.42±0.04	0.49±0.01	0.43±0.00
16:0	33.9±0.60	36.5±1.63	33.8±0.25
16:1 (n-7)	39.2±0.62	33.8±0.31	39.8±0.12
16:2(n-4)	1.03±0.03	0.80±0.02	1.02±0.02
16:3(n-4)	0.33±0.00	0.49±0.01	0.36±0.01
17:0	0.22±0.00	0.00	0.17±0.00
16:4(n-1)	0.04±0.01	0.00	0.00
18:0	1.05±0.04	2.52±0.26	1.15±0.00
18:1 (n-9)	9.20±0.26	9.31±0.42	8.69±0.04
18:1 (n-7)	1.01±0.26	2.79±0.08	0.94±0.02
18:2 (n-6)	2.70±0.02	2.16±0.08	2.70±0.03
18:2(n-4)	0.40±0.01	0.38±0.04	0.42±0.00
18:3(n-3)	0.05±0.01	0.00	0.00
18:4 (n-3)	0.02±0.01	0.00	0.00
20:0	0.13±0.00	0.46±0.10	0.16±0.01
20:1 (n-9)	0.10±0.00	0.00	0.05±0.01
20:1(n-7)	0.00	0.00	0.00
20:2 (n-6)	0.00	0.00	0.00
20:4 (n-6)	0.75±0.04	0.47±0.01	0.90±0.01
20:3 (n-6)	0.28±0.28	0.00	0.29±0.23
20:3(n-3)	0.00	0.00	0.00
20:4(n-3)	0.00	0.00	0.00
20:5 (n-3)	**6.24±0.23**	**3.90±0.23**	**6.00±0.16**
22:5 (n-3)	0.42±0.05	3.22±0.58	0.48±0.01
22:6 (n-3)	0.16±0.06	0.54±0.21	0.10±0.01
24:1 (n-9)	0.00	0.00	0.00±
Total unsaturated	61.9±1.3	57.8±1.43	61.75±0.95
Total n-3	6.8±0.32	7.66±.043	6.58±0.27

*results are presented as % of total fatty acids(n=2)

Table 5. 3 Fatty acid composition of the leaked phase from *Chlorella sorokiniana* paste which was separated by centrifugation at 4500 g.

Fatty acid	Harvested	biomass phase	leaked phase
14:0	3.23±0.28	0.55±0.03	12.7±0.11
15:0	0.44±0.04	0.12±0.03	0.48±0.08
16:0	8.63±0.43	11.8±0.04	22.9±0.09
16:1 (n-7)	10.46±0.06	11.8±0.25	6.69±0.03
Unknown	3.36±0.15	3.05±0.13	0.00
16:2(n-4)	0.35±0.04	0.11±0.05	0.52±0.03
16:3(n-4)	0.00	0.00	0.00
17:0	12.3±0.00	14.02±0.00	0.17±0.01
16:4(n-1)	2.06±0.37	1.96±0.21	0.93±0.04
18:0	0.58±0.06	0.64±0.01	1.18±0.01
18:1 (n-9)	5.37±0.21	7.93±0.01	11.00±0.21
18:1 (n-7)	0.86±0.01	0.81±0.07	6.86±0.25
18:2 (n-6)	6.22±0.11	7.46±0.40	1.76±0.04
18:2(n-4)	0.31±0.00	0.00	0.00
18:3(n-3)	40.0±0.08	37.1±0.01	2.06±0.03
18:4 (n-3)	3.00±0.03	2.63±0.45	5.25±0.30
20:1 (n-9)	0.09±0.01	0.00	0.41±0.26
20:1(n-7)	0.00	0.00	0.57±0.00
20:2 (n-6)	0.00	0.00	0.11±0.06
20:4 (n-6)	0.74±0.00	0.00	0.29±0.01
20:3 (n-6)	0.00	0.00	0.00
20:3(n-3)	0.00	0.00	0.21±0.00
20:4(n-3)	0.26±0.00	0.00	0.38±0.01
20:5 (n-3)	**1.02±0.00**	**0.00**	**15.82±0.63**
22:1(n-11)	0.00	0.00	0.47±0.01
21:5(n-3	0.00	0.00	0.23±0.09
22:5 (n-3)	0.00	0.00	0.07±0.01
22:6 (n-3)	**0.80±0.00**	**0.00**	**8.89±0.33**
24:1 (n-9)	0.00	0.00	0.00
Total Unsaturated	84.2.±0.93	81.8.±1,45	62.5.±2.31
Total n-3	44.1.±0.11	39.7.±0.45	32.9.±1.38

*results are presented as % of total fatty acids(n=2)

High concentration of EPA(C20:5 n-3) and DHA(C22:6 n-3) as value added bioactive fatty acids in the leaked phase is a benefit, as it can be separated continuously, at low RCF values with minimum damage to the microalgae.The chemical composition of the leaked phase from species mentioned above, on the other hand, represented the same composition which includes mostly lipids (more than 75%), and low concentrations of protein (**Table 5.4**).

Table 5.4 The composition of the leaked phase from *Nannochloropsis salina* and *Chlorella sorokiniana* biomass which was separated by centrifugation at 4500 g. Both cultures were harvested by SiC ceramic membrane (300mmx 0.1μm) at the room temperature and 1.0±0.1 bar pressure.

Species	*Nannochloropsis salina*		*Chlorella sorokiniana*	
	Protein(%DW)	lipids (%DW)	Protein(%DW)	lipids (%DW)
Harvested	42.2±1.8	12.34±0.40	31.5±1.23	6.30±0.40
Pink phase	2.10±0.21	83.2±0.82	4.10±0.53	74.0±0.12
Green phase	41.7±1.14	9.3±0.23	30.37±2.14	5.34±0.23

*results are presented as of %DW(n=2).

5.3.2.6 Physical properties of microalgal paste prepared by centrifugation at various RCF values

Viscosity is an important physical property of fluids for investigation of the fluid movement behaviour near solid boundaries. Viscosity in microalgae culture like ordinary liquids ranged from 0.01 to 1 Pa.s. Microalgae culture is supposed to behave as a Newtonian fluid, but, the presence of polymeric substances has been shown to make this fluid non-Newtonian (Udoman et al., 2010). Viscosity is usually independent of pressure. However, in microalgae biomass, it seems that hydromechanical forces caused by centrifugation bring the molecules significantly closer together (**Figure 5.19**) so that dynamic viscosity increased by increasing the RCF values. Both microalgae species *Nannochloropsis salina* and *Chlorella sorokiniana* showed the same pattern. Microalgae paste represents a pseudoplastic behaviour as the viscosity decreases upon shear stress and then stays constant over time. Like other non-Newtonian fluids, the relationship between the shear stress and shear rate is not constant, being affected by the shear stress applied(Belhouse, 2011).

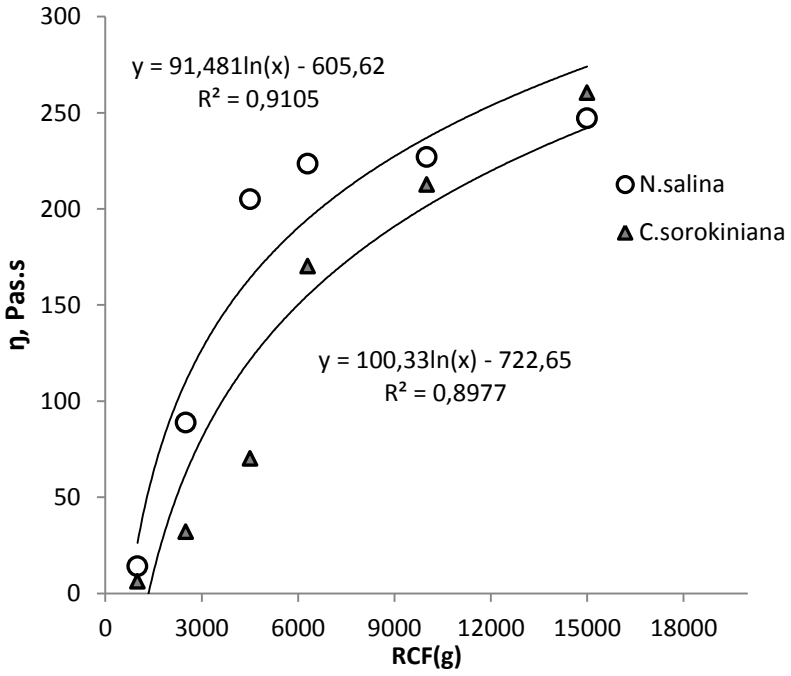


Figure 5.19. The dynamic (absolute) viscosity of algal paste prepared by centrifugation at various RCF(g). Data was collected from up-concentration trials of two microalgae species; *Nannochloropsis salina* and *Chlorella sorokiniana.*All of the samples were previously harvested by SiC ceramic membrane. Centrifugation was done at constant temperature 20 ±1℃, and for 10 minutes*(n=2).* Bars are showing the standard deviations for each data point.

5.3.2.7 Up-concentration ratio by centrifugation

The concentration ratio highly varied between harvested microalgae species. Dry matter in resulting pastes ranged from 12±2.0 % DW to 38.3 ± 1.0 %DW for several microalgae which were investigated (**Figure 5.20**). Concentration rate in microalgae paste is being affected by parameters such as packing factor, initial dry matter content in the harvested slurry, presence of polymeric substances in the liquid phase and viscoelastic property of the paste (Willeman et al., 2014). As shown in **Figure 5.20**, the initial concentration of dry matter for *Chlorella vulgaris* was higher than for *Nannochloropsis salina*, but the dry matter obtained at higher RCF values was apparently lower for *Chlorella vulgaris*. The packing factor, Ø is defined as the volumetric proportion of solid to the liquid in the paste. Both average cell size and cell shape affect the packing factor (Souli`es et al,. 2013). For *Chlorella vulgaris*, with comparatively big cell size ( 7-9 μm), The maximum packing fraction was reported as Ø= 0.637 for a dry matter concentration of 159.25 g/L (Souli`es et al., 2013). On the other

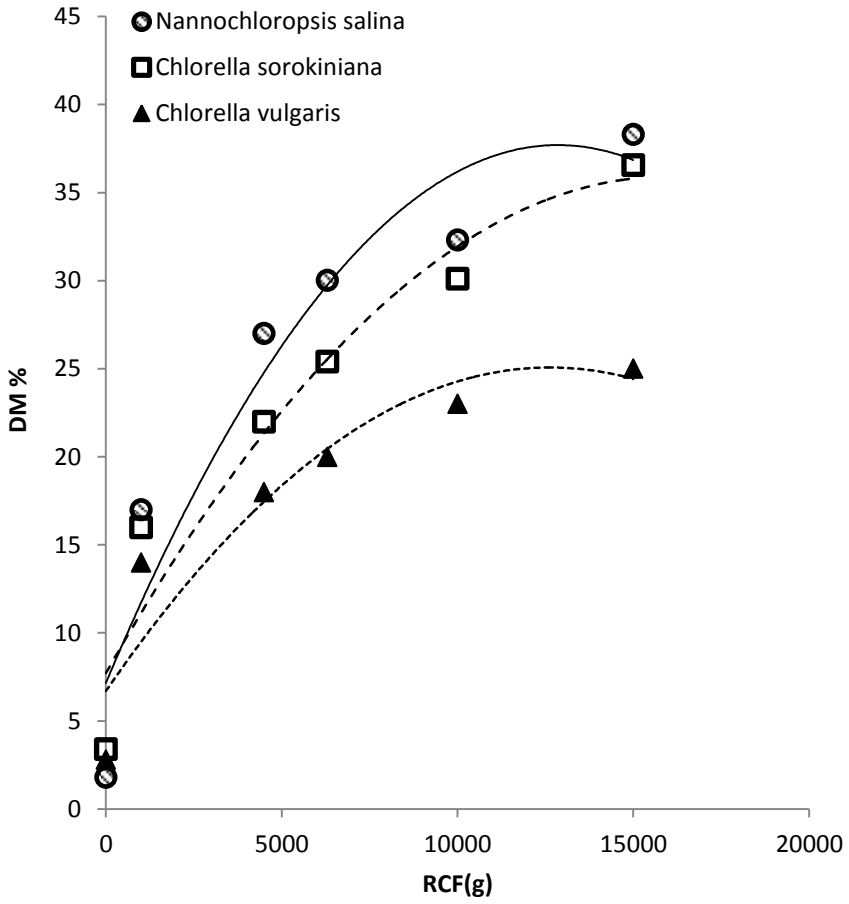


Figure 5.20. The dry matter content in algal pastes prepared by centrifugation at different RCF(g). Data was collected from up-concentration trials of three microalgae species; *Nannochloropsis salina*, *Chlorella sorokiniana and Chlorella vulgaris.* All of the samples were previously harvested b SiC ceramic membrane. Centrifugation was done at constant temperature 20 ±1℃, and for 10 minutes.

hand, microalgae such as *Chlorella vulgaris* can produce extracellular polymeric substances under stress condition and to overcome the mechanical stresses such as hydrodynamic forces. These compounds bond the water and hence, the separation of the bonded water would require very high centrifugal foirces so increasing the centrifugation force did not alter the dry matter concentration in the biomass. For two other species, higher dry matter content was achieved at elevated RCF values. Displacement of the resulting paste, with 35±4% dry matter and reasonably high viscosity, requires a positive displacement pumping system. Willeman et al. (2014) demonstrated that the bioenergy pumping effectiveness increases linearly at a rate of about 46 units per kg/m3 in relation to the biomass concentration. In other words, increasing the biomass concentration do not increase the pumping costs, as the pumping efficiency increases. As a conclusion to this part; centrifugation of the

harvested microalgae can be done by a tubular bowl centrifuge to prevent the high shear caused by the elevated RCF's which normally occur in disc stack centrifuges. The optimum range of 6500±500 g can be recommended to avoid the cell rupture due to the centrifugal forces.

5.3.3 Heat treatment of microalgae slurries

Microalgae contain enzymes such as lipase and lipoxygenase, which could degrade the cellular lipids, especially in the wet biomass (Chen et al., 2012). Krohn et al. (2011) found that free fatty acid levels in extracted oil increased very fast to values as high as 84%, which was due to the presence of endogenous enzymes such as lipase in the microalgae cell. Inactivation of the enzymes by a heating process could extend the shelf life of lipid-containing microalgae biomass. On the other hand, the presence of sensible heat compounds such as carotenoids and highly unsaturated fatty acids, and the energy consumption of the heat treatment make the process tricky. A gentle heat treatment such as pasteurisation, which deactivates the milk enzymes with less adverse effects on the sensible bioactive compounds can be suitable to the microalgae. Several heat-treatment regimes, i.e. various combinations of temperature & time were tested on chlorella sorokiniana biomass in preliminary studies (data are not shown here). The microalgae harvested by SiC ceramic membrane microfiltration was used for all experiments. The combination of 75℃ and 15 seconds was selected due to less deterioration of heat sensitive carotenoids and the enzyme inactivation. The effects of a similar heat treatment on the fatty acids and pigment composition of Nannochloropsis salina were evaluated (**Figures 5.21 and 5.22**). As shown in Figure 5.21, just 7.8% reduction in the contents of eicosapentaenoic acid (C20:5 n-3) was caused by the heat treatment. The contents of the carotenoids was also affected by the heat treatment process (**Figure 5.22**), which surprisingly demonstrated higher levels of violaxanthin in the heated sample. This result can be justified with the fact that heat treatment could affect the cell wall and enhance the extractability of the pigments. On the other hand, changes in the concentrations of pheophytin and other chlorophyll degradation products are negligible, which demonstrate that the heat treatment did not alter the chlorophylls. The reduction of eicosapentaenoic acid can be justified by the fact that this fatty acid is present in the cell wall membranes as shown previously (**Table 5.3**).

On the other hand, the lower content of lipids and higher contents of the protein in the heat treated sample (data are not shown here) which confirms the cell rupture, which resulted in higher extractability of these compounds. An experiment storage trial was also done to evaluate the

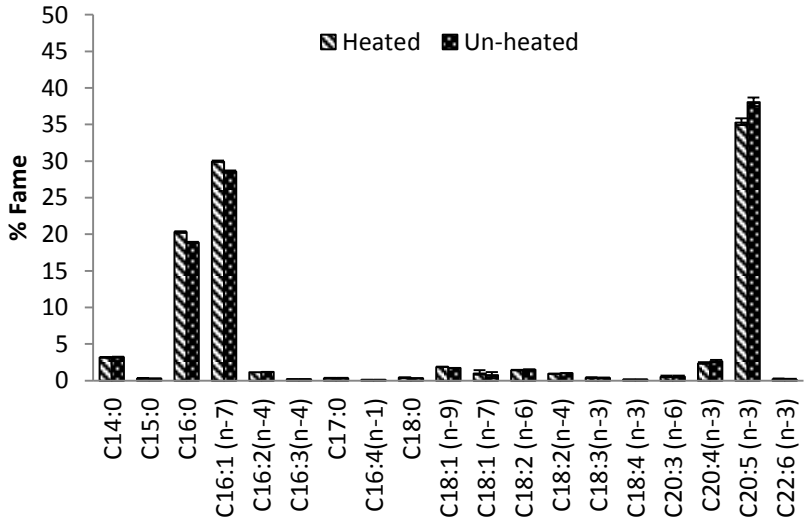


Figure 5.21 Effect of heat treatment (75℃&15 seconds) on the fatty acid composition of harvested biomass slurries from *Nannochloropsis salina*. Bars show the standard deviation(n=2).

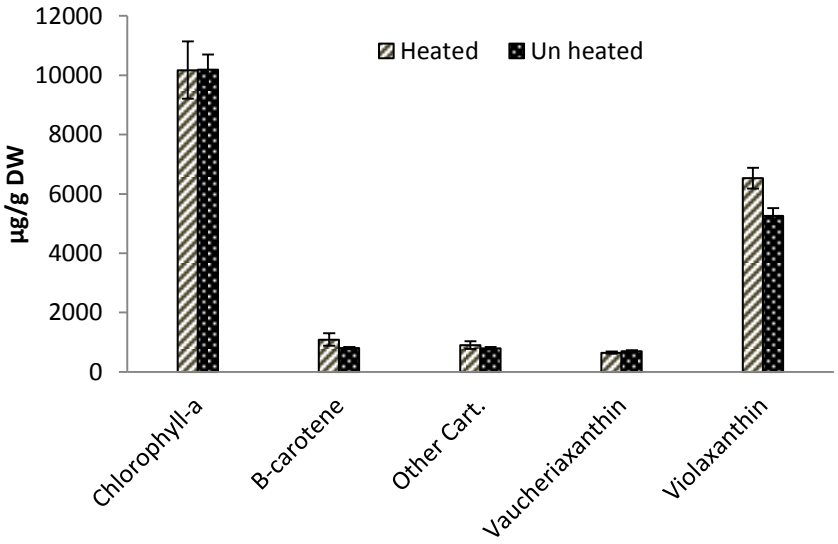


Figure 5.22. Effect of heat treatment (75℃&15 seconds) on the pigments composition of harvested slurries from *Nannochloropsis salina*. Bars show the standard deviation(n=2).

consequences of the heat treatment on the development of volatiles. Results of the analyses are shown in **Figure 5.23**.

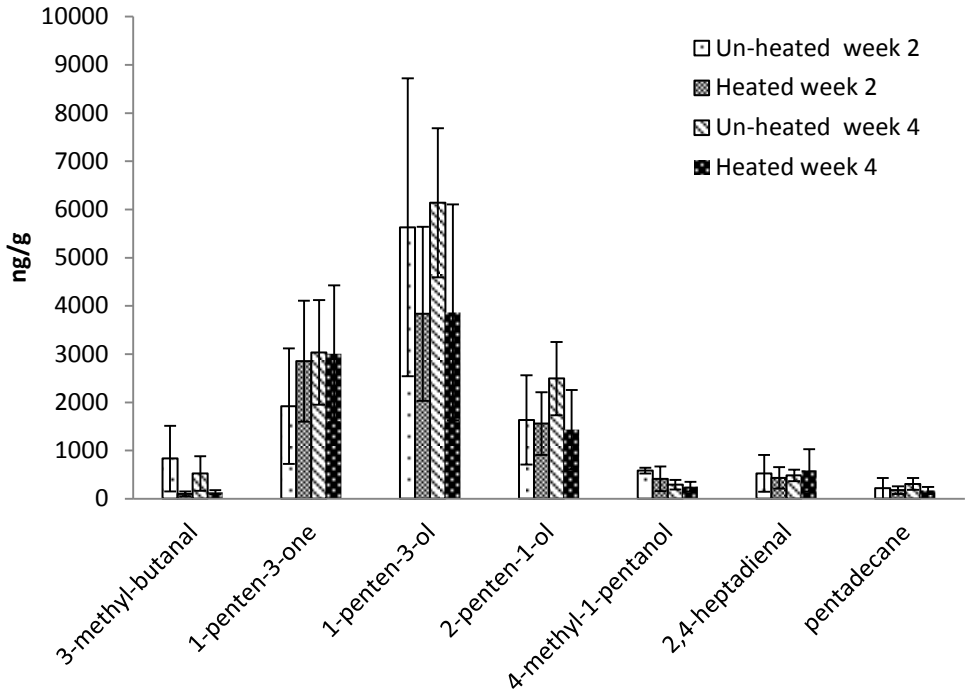


Figure 5.23 Effect of heat treatment (75 ℃ & 15 seconds) on the formation of volatiles of harvested slurries from *Nannochloropsis salina*. Bars show the standard deviation(n=3). Samples packed under vacuum and stored at 20±2℃ prior to the analysis.

During the storage time, the samples were kept under vacuum, because heat treatment cause rupture to the microalgae cell and liberation of the intercellular lipids to the oxygen accelerate the oxidation, so the effect of enzyme activity may not be evaluated. As shown in **Figure 5.23**, the contents of 1-penten-3-ol and 3-methyl-butanal were lower in the heat treated samples. Amounts of 2-penten-1 ol were also higher in unheated sample after 30 days of storage. Variations in the other volatiles were not different during the storage. The results offer that heat treatment of microalgae biomass inactivates the endogenous lipase while rupture the membrane in some extent, so the digestibility and the extractability of the nutrient might be improved. It should be considered that trough the heat drying methods, such as spray drying, drum drying or cabinet drying, the enzymes are being inactivated due to the high temperature(more than 100 ℃), so the heat treatment would be required when a gentle drying method such as freeze drying is applied. Heat treatment also causes physical deterioration to the algae paste (**Figure 5.24**), which can be justified with the cell rupture and partial protein coagulations resulting from the heat. Heat treated algal paste lose the viscoelastic property because of

the destruction of extracellular membrane charges so that the texture behaviour turned to, grainy articulated semi-solid.

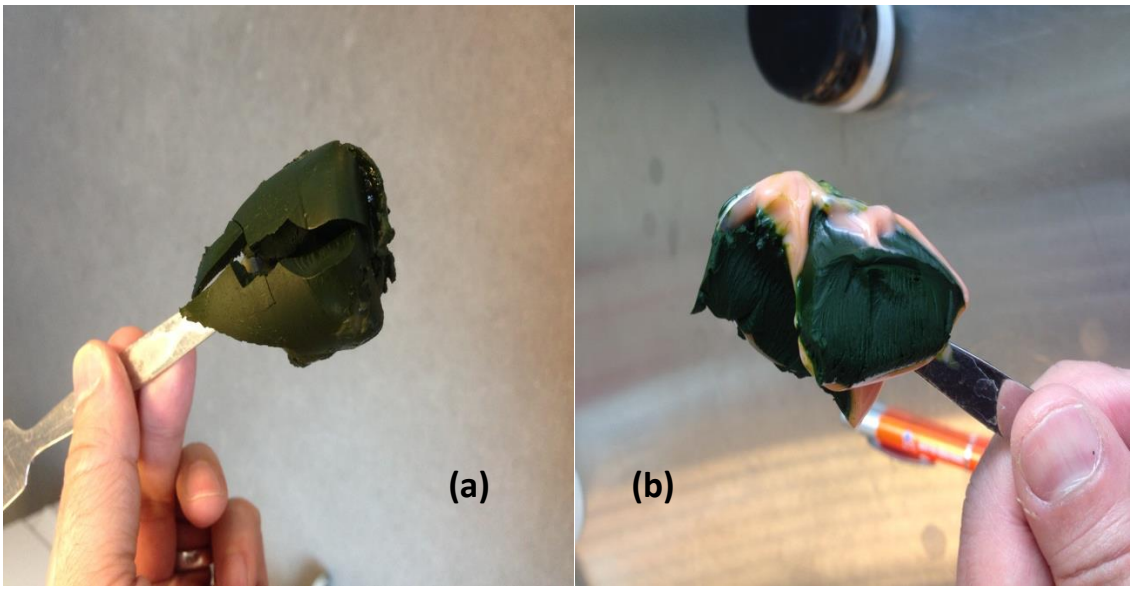


Figure 5. 24 Effect of heat treatment (75°C&15 seconds) on the physical appearance of a *Chlorella sorokiniana* paste, (**a**); heat treated and (**b**) not heated pastes Centrifugation was done at 6300 g for 10 minutes.

Heat treatment is a recommended process when the drying is being done by gentle drying methods such as freeze drying.

5.3.4 Drying of microalgae biomass

Drying of microalgae is required to facilitate the transportation, extend the shelf life and storage ability and to ease the applications such as food and feed formulations. Drying is the last part of the downstream processing, requires large amounts of energy and involves irreversible deteriorations to the product. The drying technology is specific for every product, so a dryer designed for a specific application may not function correctly for other purposes. There is no single design procedure which

can cover all specific applications. The design of a specific dryer for microalgae requires practical data out of pilot scale experiments. Factors affecting the selection of a drying technology include the physical status of the feed (powder, paste, high or low liquid), initial and desired final moisture content, the application of the end product, quality requirements and finally the maximum resistive temperature when the feed includes sensible heat compounds. In our process design, water is removed as much as possible, using physical methods which require no heat so that the bioactive compounds do not during the harvest and up concentration. The resulting high viscosity paste includes live organism (to some extent), 60-70 % moisture and nutrient so that to prevent the microbial deteriorations; it should be dried very fast. On the other hand, drying of paste-like materials such as pastes, suspensions, slurries and emulsions are hard (Kudra et al. 2007). Drying technologies which could be applied to the paste-like materials include; tray drying, kiln dryer, rotary dryer, screw conveyor dryer, steam tube rotary dryers, pneumatic dryers, fluidised or spouted bed dryers. Drying of microalgae requires very high contact between the drying gas and the product particles so that the drying occurs very fast. Such great contact area could be provided by fluidised bed, flash drying or spray drying techniques. Spray drying could not be used for the drying of microalgae paste because of the very high viscosity of the feed (Katie, 2000). On the other hand, fluidised bed dryers could only operate on powders or granules. Results of the laboratory scale studies with a prototype flash dryer revealed that the microalgae paste requires a dispersion method to distribute the feed in small particles in the stream of drying medium. Otherwise the feed sticks to the walls of the drying tube, so that the drying would not be efficient. The spin flash drying technology was finally selected, which in theory is a combination of fluidised and flash drying (Wang et al. 2004), while this technology has never been used for the drying of microalgae. This technique was developed and introduced in 1970 as a response to the chemical industry needs for a drying method to handle high viscosity fluids, cohesive pastes and sludges (Katie, 2000).The initial data and the specification of dryer prototype are shown in **Table 5.5**.

5.3.5 Drying process description

Due to the physical status of the feed as a non-Newtonian pseudoplastic fluid, a feeding unit (**Figure 5.25a**) introduces the feed to the drying chamber, which immediately is scraped- distributed by the scraping paddles (**Figure 5.25b**), which moves close to the drying chamber walls (2mm) and fluidised in the stream of the air. After drying, which occurs in seconds, the density of smaller particles reduces so that they can be moved with the stream of air to the outlet. Bigger particles return continuously to the scrapping area and can move to the outlet and are being distributed to smaller sizes. The air inlet, located at the conically shaped bottom of drying chamber, so the air stream flows spirally to the drying chamber, prevents the feed from sticking on the walls.

Table 5.5 Initial data for ;(a) drying of microalgae paste; and (b) specification of prototype dryer.

Parameters	Range	Unit
Feed rate	2-5	kg/h
Feed moisture content	60-70	% WB
Feed viscosity	25-50	Pa s
Feed density	1150-1200	kg m^{-3}
Product particle size	200-500	μm
Drying gas temperature	100-150	°C
Humidity of inlet air	0.002	kg/kg air
Air density	0.7	kg/m^3
	(b)	
Parameters	Range	Unit
Evaporation capacity	max 3.5	kg/h
Drying tube length	100	cm
Drying tube diameter	120	mm
Feed density	1150-1200	kg m^{-3}
Air flow rate	max 120	m^3/h
Drying gas temperature	100-150	°C
Scraping paddle speed	max 1000	rpm
Overall velocity	max 3	m/s

5.3.6 Effect of the feed moisture content on the required drying air volume

Based on the drying model, the required drying air volume increases when the moisture content of the feed increases. Successful drying of microalgae pastes with higher moisture content (80-90%), requires high temperatures but elevating the drying air temperature to a degree more than 150℃ results in product burning. To overcome this problem, increasing the drying air volume increased the drying rate. While in practice because of co-effect of agitating paddles, the required air volume reduced to 20-30%.

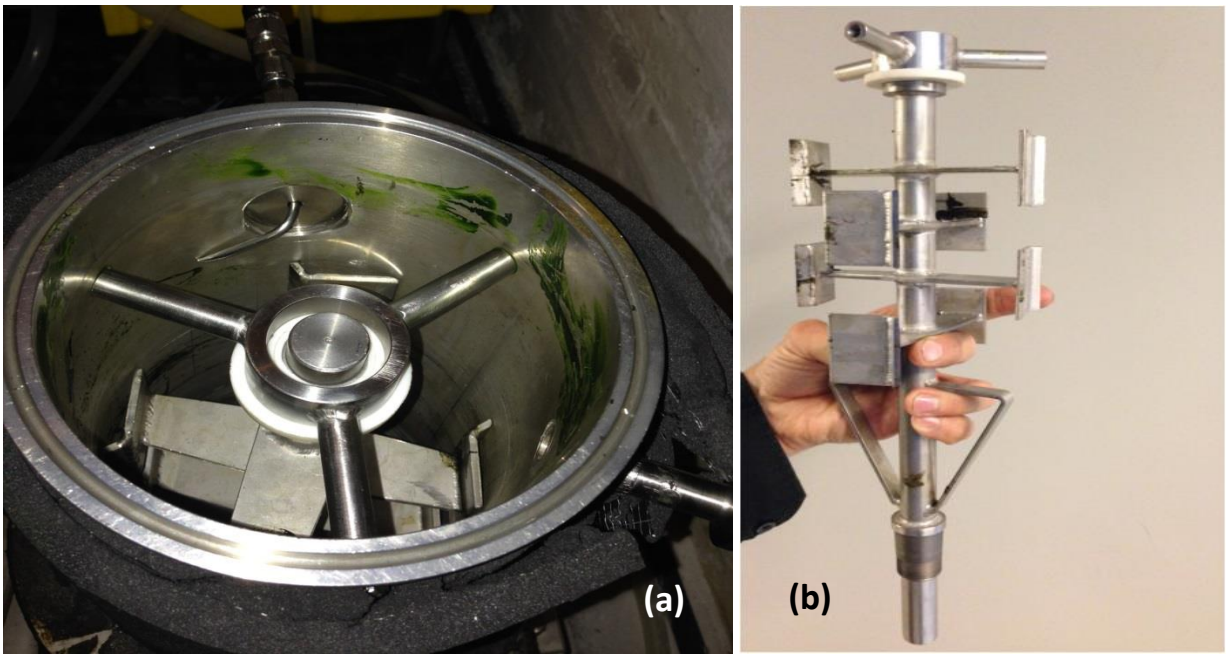


Figure 5.25 Schematic of feeding(a), and scraping system(b) in the dryer.

5.3.7 Distribution of the air velocity in drying tube

The air velocity in drying chamber includes tangential velocity, axial velocity and the radial velocity (Wang et al., 2004). Tangential velocity is more important as it moves the particles toward the walls to the outlet.Tangential velocity is a function of both paddle rotating speed and the airstream. Because of the air friction, tangential velocity decreases when the drying tube length increase. On the other hand, centrifugal force caused by the moving paddle is the highest in the area close to the walls (**Figure 5.26**) so reduction of the drying tube diameter provides a more homogenous air stream

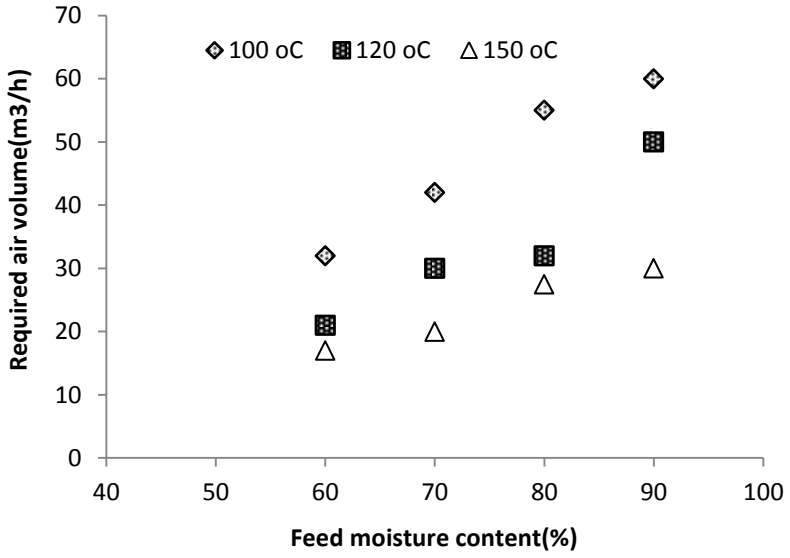


Figure 5.26 Effect of Feed moisture content on required air volume at various Air temperatures.

5.3.7 Distribution of the humidity in drying tube

Distribution of particle sizes and humidity along the drying tube is shown in **Figure 5.27** and **5.28** Drying temperature was 120 ℃, with an air velocity 1.6 m/s (overall velocity 2.8 m/s) and initial moisture content of the paste was 83±2.0%. As shown, moisture content decreases along the drying tube, so that for a paste with very high moisture content, either the drying length or the drying temperature shall be increased. In a swirl flash dryer movement and distribution of particles are influenced by tangential, axial, radial velocity. As algae paste, especially when the moisture content is high, may stick to walls of drying tube, so that the tangential velocity should be high enough to accelerate the drying and reduce the risk of product burning on the hot walls. Bigger particles moved to the side walls, while the smaller particles(e.g. 100 μm) directly move up in the centre. Wang et al. (2004), suggested that the particle diameter in a swirl flash dryer can be estimated from the equation:

$$d_p = \frac{18\mu}{h\omega^2(\rho_s - \rho)} \; Ln\frac{r}{r'} \cdot \frac{v}{\pi R^2} \qquad (6)$$

Where; μ is air viscosity; ω is the particle angular velocity; $\rho_s$is the particle density; ρ is the gas density ; r is the distnce from partcle to the center of drying chamber; r' is rotary radius of te particles at the bottom of drying chamber; v is the velocity and R is the chmber diameter.

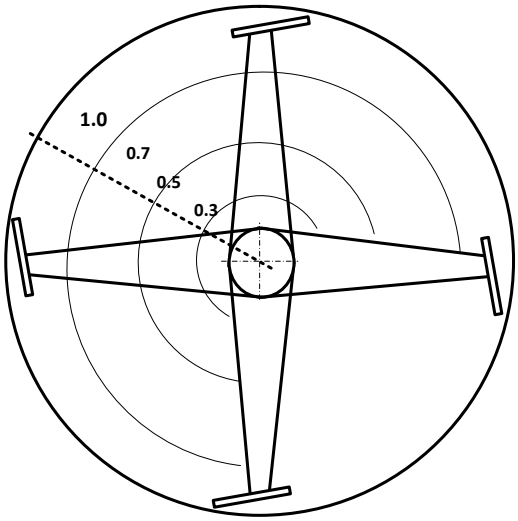


Figure 5.27. Flow (as velocity in m/s)pattern in the drying chamber, across the drying tube diameter and (b) distribution along the drying tube height. Measurements were done at 20 cm above the paddles.

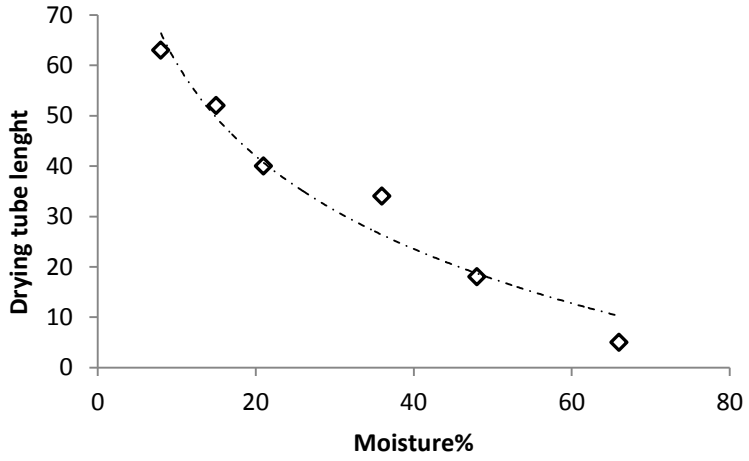


Figure 5.28 The moisture content of microalgae pastes along the drying tube length. The overall velocity was v=2.8 m/s; the temperature of drying air=120℃.

The equation was presented to estimate the particle distrbution pattern in the drying chamber. While the particle distribution range in the microalgae drying trials was high, based on practical dring trials

the above mentioned equation could not predict the distribution along the drying tube area. The relationship between the terminal velocity (V_t) and minimum fluidizing velocity(V_{mf}) to the particle size is shown in **Figure 5.29.**

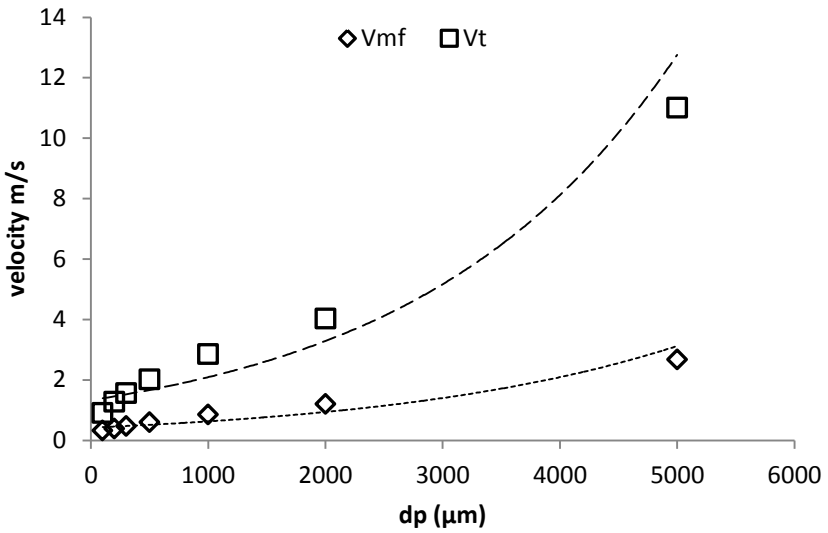


Figure 5.29 The relationship between the terminal velocity, minimum fluidising velocity and particle diameter

However, the non-spherical shape of the particles of microalgae biomass and a huge size deviation makes the estimation erroneous. Control of the particle carries over trough the system is possible by having control of the rotation speed of the scrapping paddles and the flow rate of drying air. Our experiments revealed that overall velocity as a combination of tangential and axial velocity could be 2-4 times higher than axial flow, depending on the air humidity, temperature, and the value of axial velocity by itself.

In **Figure 5.30**, Reynolds numbers as a function of particle size are shown. These practical data are critically important for the estimation and modelling of drying process. In fluid mechanics, the Reynolds number (Re) is a dimensionless number that gives a measure of the ratio of inertial forces to viscous forces and consequently quantifies the relative importance of these forces for given flow conditions**.**

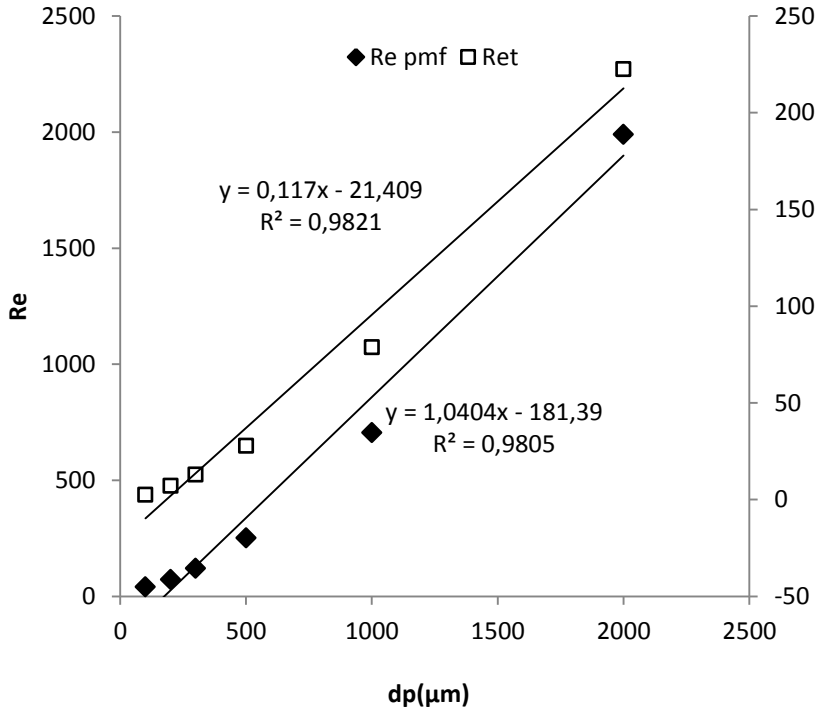


Figure 5.30 Reynolds number as a function of particle size.

5.3.8 Effect of drying on the quality of microalgae powder

Initial drying conditions were optimised during the preliminary studies, then some drying trials were done on a *Nannochloropsis salina* paste. The drying conditions are shown in **Table 5.6.**

In **Figure 5.31a**, effect of drying on the fatty acid composition was shown. Surprisingly, the reduction in the content of sensitive heat eicosapentaenoic acid was very low, which could be justified by the low drying time. Analysis of the pigment composition also revealed (**Figure 5.31b),** that the drying has no adverse effect on the sensible heat carotenoids such as violaxanthin. Higher concentration of the pigments in the dried microalgae sample can be justified by the fact that the extractability of the pigments was improved due to the heat-induced cell rupture during the trial. It can be presented as an advantage compared to the spray drying, which is the most common method of the drying of microalgae in which the quality of value added carotenoids are being destroyed, while the chlorophylls also convert to the pheophytin type products which are known as powerful photo peroxidant, reduce the storage stability of the powder.

Table 5.6. Drying condition used at the pilot scale trials.

Parameters	Range	Unit
Feed rate	1	kg/h
Feed moisture content	74.5	% WB
Feed viscosity	25-50	Pa s
Product moisture content	8	% WB
Product particle size(estimated)	200-1500	µm
Drying gas temperature	120	°C
Humidity of inlet air	0.003	kg/kg air
Air density	0.7	kg/m^3
Drying time	3.3	sec
Air outlet temperature	68	°C
Air velocity	2 8	m/s
Volumetric air flow rate	18	m^3/h

5.3.9. Energy consumption

From an economical point of view, swirl(spin) flash dryer uses less energy per kg weight of dried product, require less investement and even less area for the installation and application, when compared to spray drying in the same condition (Katie, 2000; Pertick et al., 2013). The cost per unit of the drying by this technique was estimated to be 28% less than spray drying for the same product (Katie, 2000). The dryer design is complex, but the construction do not require special high tech units such as atomizer in spray dryer. On the other hand this system could be operated in small scales which is desired for the microalgae cultivation systems, which produce between 10-100 kg of the paste per day. This system can handle the high viscosity microalgae paste in which the main part of the water is removed by means on mechanical methods, including micro filtrationand centrifugation. So that less heat energy is required for the drying whih equals to the lower degradation of valuable compound in the microalgae biomass. The prototype swirl flash dryer dsigned for the specific application of microalgae drying, while continual improvement would always be required.

The process set up include the cross flow microfiltration by SiC (0.1µm) ceramic membranes, then up concentration by bowl centrifuge at 6500±500 g and finally drying by the the swirl(spin) flash dryer which designed specifically and tested on microalgae samples. Energy consumption per kg of the product was evaluated as 2.2 KWh. In **Table 5.7**, the energy consumption by the invented method is compared to a common commercial method (spray drying). It could also be pointed out that the

deterioration of, value-added bioactive compounds such a carotenoids is less in the invented method, as it was shown previously.

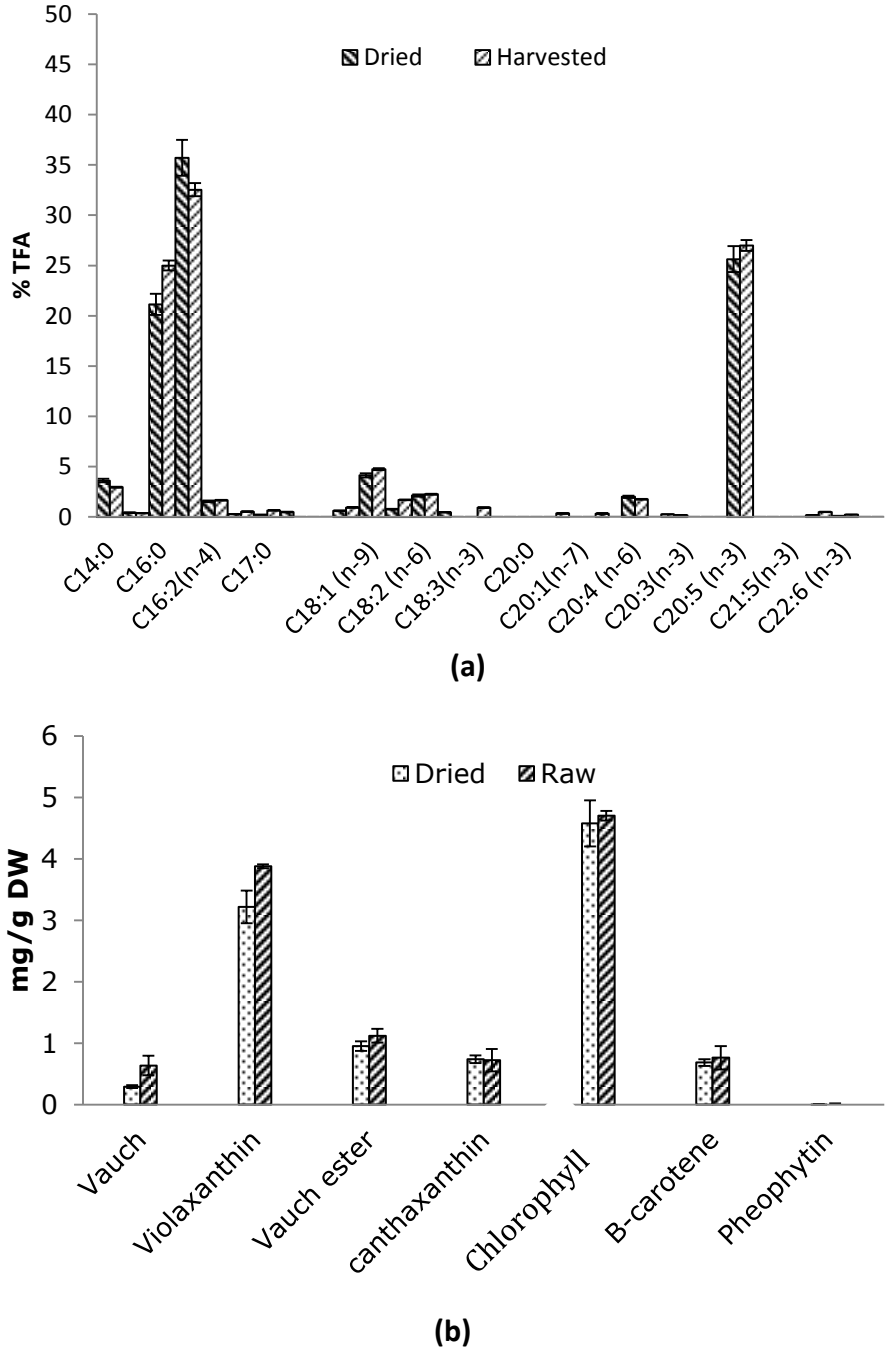


Figure 5.31. Effect of the drying by swirl flash dryer on (a) fatty acid composition and (b) pigment composition of *Nannochloropsi salina* biomass.

Table 5.7. Typical energy consumption of the novel downstream processing method (scenario 1), compared to the current commercial spray drying method (scenario 2). The harvest method (Membrane filtration)was the same in both scenarios.

Capacity of each Flat panel photobioreactor	4000 L
Number of photobioreactors	8
Daily harvest (25% of total volume)	8000
Energy consumption of harvest by microfiltration(2-3 kWh/m^3)	20 kWh
Yield (average 25 g/L DM)	500 L

Scenario 1; Up concentration-Drying	
Up concentration by centrifuge;	
Yield(250-350 g/L DM)	50 L
Energy consumption of up concentration(5-7 kWh/m^3)	2.5-3.5 kWh
Drying by swirl flash dryer;	
Energy consumption of drying(2.2 kWh/kg DW)	37.5 kWh
Yield(995 g/L DM)	15 Kg
Total energy consumption (kWh/kg DW)	60-62 KWh

Scenario 2; Direct drying	
Drying by spray dryer;	
Energy consumption of up drying(3.5 kWh/kg DW)	72.5-75 kWh
Yield(995 g/L DM)	15 Kg

5.3.10. Conclusions

The swirl flash drying technique was successfully used for the drying of a microalgae biomass from *Nannochlorosis salina*. The design of the prototype dryer was done based on the data from preliminary studies on the physio chemical and drying charachteristics of the microalgae biomass. The dryer is specifically designed to handle microalgae paste as a feed. Analysis of the pigment and fatty acid composition also revealed that the drying technique had low adverse effects on the quality of

microalgae biomass, which comapared to the other methods such as spray drying was very promising. To our knowledge this technology was never used for the drying of microalgae. The design of the dryer was also application specific.

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Chapter 6; Effect of storage conditions on the oxidative stability and nutritional composition of freeze-dried *Nannochloropsis salina*

6.1. Introduction

This chapter gives an overview on the storage stability of freeze-dried microalgae biomass which is presented in details in paper 6. Application of microalgae biomass in food and feed products requires information regarding storage stability and optimised storage conditions to minimize unwanted deterioration which downgrades the biomass. The information about variations of the valuable compounds during dry storage of microalgae biomass is demonstrated in a few studies.

6.2. Materials and methods

The algae *Nannochloropsis salina* was cultivated on an ICW enriched growth medium in flat panel photobioreactor as described in paper 3. The biomass was selected due to the high contents of tocopherols, eicosapentanoic acid (C20:5 n-3, EPA) and carotenoids (paper 3 and 5). The harvest and up concentration was done in large scale by microfiltration and centrifugation techniques, respectively and the resulting paste was freeze dried immediately to a water content less than 10% (w/w). A multifactorial storage experiment was conducted to investigate the variation of the nutritional quality of freeze dried biomass. The storage time (0 to 56 days), storage temperature (5℃, 20℃ and 40℃) and packaging conditions (under vacuum and ambient pressure) were used as main factors as it has shown in **Figure 6.1**.

Total lipids of all collected samples were extracted with chloroform/methanol according to the method described by Bligh&Dyer. Analysis of tocopherols was done by HPLC-FLD, according to AOCS official method. The fatty acid composition was determined by GLC-FID as described in paper six. Pigments were analysed by HPLC-DAD using the method described in paper 1. Peroxide value (PV) and free fatty acids (FFA) of the Bligh & Dyer extracted lipid fraction was determined according to the methods described in paper 6. The analysis of volatiles was done by a dynamic headspace procedure using the Tenax tube. The separation, identification and quantification of volatiles was done by GC-MS as in Chapter 5. See paper 6 for details.

6.3. Results and discussion

6.3.1. Lipids and EPA

Storage time had statistically significant effect on the contents of lipids at the 95.0% confidence level (P-value <0.05). Probably the activity of enzymes which takes place at the first days of storage (Balduyck et al., 2015) increased the liberation of lipids and as a result, extractability of the lipids at the first sampling time was higher. On the other hand, cell rupture which occurs during freeze drying of microalgae may result in the same.

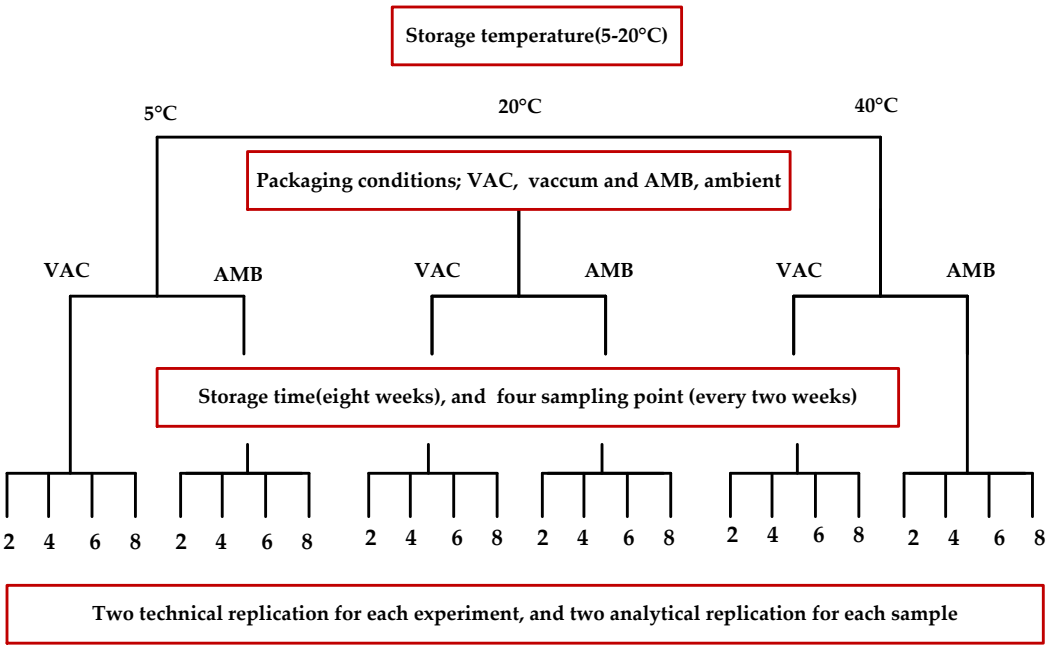

Figure 6.1 The experimental design of the study included three factorial levels as factor 1(the storage temperature; 5, 20 and 40°C), factor 2 (packaging conditions; under vacuum or ambient pressure), and factor 3 (the storage time; eight weeks).

Variations in contents of EPA were found to be influenced by both storage time and packaging conditions. The lowest and highest loss in EPA contents after 56 days of storage were observed for 5°C-VAC and 40°C-AMB experiments as -12.48 % and -15.87%, respectively. The EPA content declined more notoriously during the first weeks of storage (between days 0 to 14) and then continued to decrease during the time, albeit at a lower rate. There are various classes of natural antioxidants in the microalgae biomass, including tocopherols and carotenoids which mostly remain intact during the freeze drying. These natural antioxidants might inhibit or retard the lipid oxidation induced EPA loss. However, this process results in their own decomposition (Balduyck et al. 2015). Hence, it cannot be ruled out that the presence of these antioxidants prevented a faster decomposition of EPA, particularly after two weeks of storage.

6.3.2. Natural antioxidants

Tocopherol (α-tocopherol) contents were significantly influenced by the storage time and packaging conditions. The lowest and highest losses were observed for 5°C-VAC and 40°C-AMB experiments as -14.57% and -67.92 %, respectively (Table 6.1). These results strengthen the idea that α-tocopherol, as a natural antioxidant, might have been involved in the protection EPA form oxidation

deterioration, by scavenging of oxidation inducing radicals at the expense of its own decomposition. It is expected that EPA content will decline more drastically when the α-tocopherol has been entirely ruined.

The contents of carotenes drastically declined at first 14 days of storage. The highest loss was measured in the samples stored at 40℃ (-94.08% for 40℃ – AMB) which was significantly greater compared to the samples stored at 20℃ (-76%), and 5℃ (- 69%). Temperature sensitivity of carotenoids depends on their chemical structure. Carotenoids with a longer conjugated carbon-carbon double bond structure (e.g. β-carotene) are more heat-sensitive (Ryckebosch et al., 2011). The vacuum packaging did not improve the stability of carotenes in the samples stored at higher temperatures. Moreover, xanthophylls (mainly include free/ esterified vaucheriaxanthin, violaxanthin, canthaxanthin and minor compounds such as diadinoxanthin) also declined during the storage time. The storage temperature and the interaction of time-temperature significantly influenced the total xanthophylls. Effects of the packaging conditions and its interactions were evaluated as not significant ($p > 0.05$). Total xanthophylls declined in all samples, particulary during the first 14 days. The reduction rate was faster for the samples stored at 40℃ followed by 20 ℃ and then 5℃, respectively. It was somewhat surprising that vacuum packaging did not reduce the rate of decomposition of xanthophylls. These findings are in agreement with the results obtained by Tang and Chen (2000). Similar to the tocopherols, carotenoids, which are known as strong antioxidants, may retard the oxidation induced changes in EPA. A clear correlation between the disappearance of carotenoids and the inhibition of lipid peroxidation was also suggested previously (Ryckebosch et al., 2011). Carotenoids, α-tocopherol and other natural antioxidants in microalgae biomass (paper 1) prevent/retard the oxidative damages to the unsaturated fatty acids such as EPA during the storage.

Table 6.1. α-Tocopherols contents (μg/g of the sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $p < 0.05$.

	5℃		20℃		40℃	
Time(day)	**VAC**	**AMB**	**VAC**	**AMB**	**VAC**	**AMB**
0	460.5±67.1 bc,w,p	460.5±67.1 c,w,p	460.5±67.1 b,w,p	460.5±67.1 c,w,p	460.5±67.1 b,w,p	460.5±67.1 c,w,p
14	411.7±63.3 b,x,q	310.2±113.6 a,w,p	452.9±15.8 b,x,q	386.5±29.9 b,wx,p	305.8±90.2 a,w,p	302.2±15.3 b,w,p
28	308.6±61.6 a,wx,q	264.2±7.67 a,w,p	525.1±94.1 bc,z,q	342.2±22. 3 ab, x,p	494.8±86.1 bc,y,q	291.1±49.1 b,w,p
42	415.2±82.8 b,x,p	409.8±4.81 bc,x,p	434.75±11.2 b,x,q	316.39±12.7 a,w,p	437.5±44.1 b,x,q	265.1±49.3 b,w,p
56	393.4±56.2 ab,y,p	351.6±12.5 ab,xy,p	362.76±8.25 a,xy,p	297.98±34.5 a,x,p	313.11±9.24 a,x,q	147.7±43.9 a,w,p

6.3.3. Oxidation and lipolysis

In this study, storage time and the interaction of temperature- packaging conditions significantly affected the absolute change in peroxide value (PV) compared to time 0 (**Figure 6.2**).

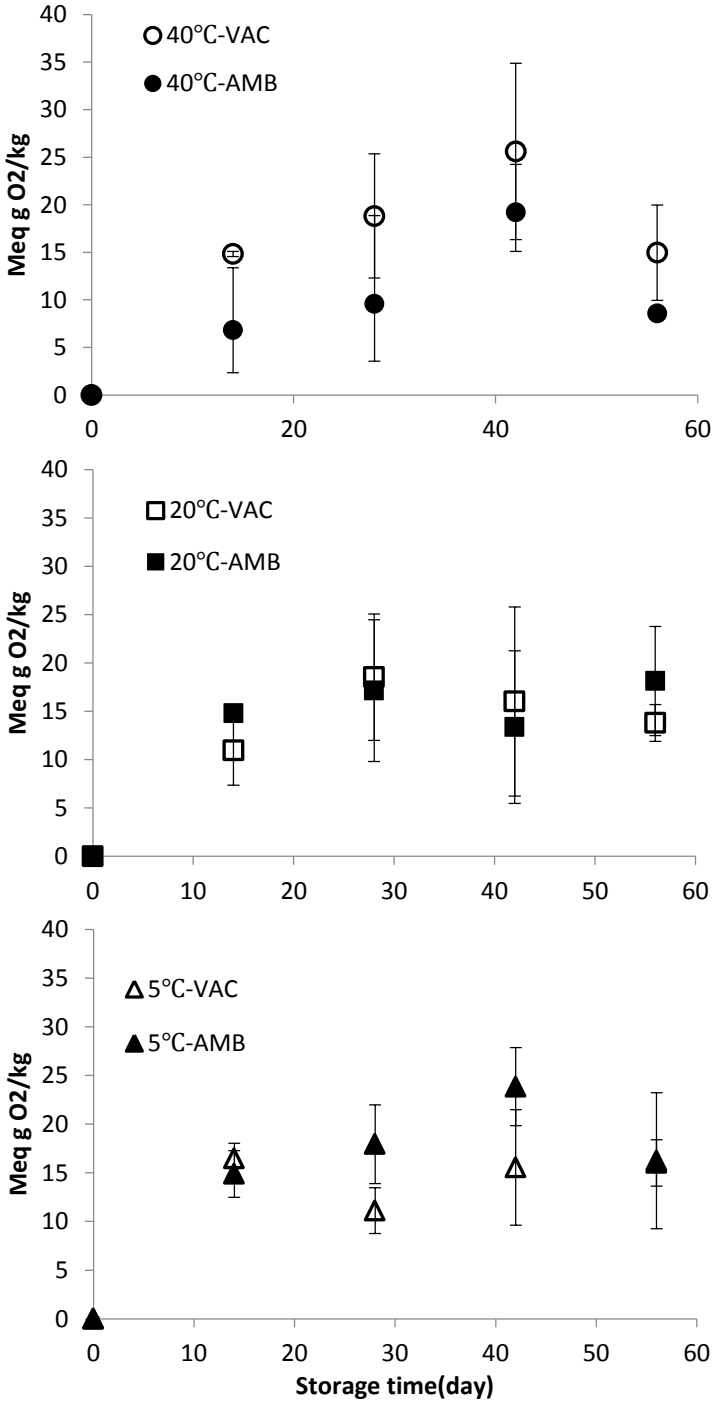

Figure 6.2. Absolute changes in peroxide values compared with day 0 (Meq g O2 /g lipids DW; mean ± SD; n = 4) as a function of storage time, storage temperature and Packaging conditions.

Hydroperoxides are unstable products, which decompose to several, mostly volatile compounds such as aldehydes and ketones. Hydroperoxide formation and decomposition involve a very complicated set of reaction pathways as it was shown in several studies (Frankel, 1980). On the other hand, pigments and individual carotenoids which were present in microalgae biomass may interfere in the absorbance spectra when colorimetric method like this are used. For this reason, the values reported here were obtained by deducting the observed PV from the PV at day 0.

The same problem was declared for the measurement of PV in microalgae biomass (Ryckebosch et al., 2013). Enzyme-induced lipolysis has been suggested to be the biggest problem for the wet storage of microalgae. This activity results in the liberation of free fatty acids which are more sensitive to oxidation. The enzyme activity declines in the dried microalgal biomass (Babarro et al., 2000). Freeze-drying does not destroy the enzymes, and because the optimum activity temperature for lipase is 30-40°C, the storage temperature might influence the free fatty acid contents in the microalgae biomass. The initial amounts of free fatty acids in the biomass was high (17.2±1.20% total lipids). Compounds such as pigments and organic acids may interfere with the results, as a basic titration method was used in this study. Therefore, confirmation of the results was made (data are not shown here) by solid phase extraction of lipid classes of the extracted lipids, and subsequent quantitative determination of fatty acids as described by Kim and Salem (1990).

The high content of free fatty acids in freshly harvested microalgae has already been reported in several studies (Olofsson et al., 2012; Balduyck et al. 2015). Microalgae probably produce extracellular free fatty acids, either to attract the bacteria or as a defence mechanism against the grazers, so the initial free fatty acid contents of microalgae are high, especially for the algae harvested at autumn-winter (Olofsson et al., 2012). It may also be attributed to the cell rupture caused by the high shear centrifugation and/or freeze drying which were used for the preparation of the algae powder. Both of storage temperature and time significantly influenced the variations of free fatty acids during the experiment. However, higher contents of free fatty acids observed in samples stored at 40℃ and 20℃, compared to 5℃, as enzyme activity at 5℃ are lower than 20℃ and 40℃.

Various volatile compounds may be produced by biological or chemical reactions in microalgae biomass (Milovanovic et al., 2015). These volatile compounds include hydrocarbons, terpenes, phenols, alcohols, aldehydes, ketones, esters and halogen or sulfur-containing compounds. The short chain linear aldehydes are often derived from chemical lipid oxidation while branched and aromatic aldehydes are typically formed due to enzymatic lipid and protein oxidation (Van Durme et al., 2013). In addition to propanal, as a quantitatively important volatile deriving from autoxidation, 1-Penten-3-one, 1-penten-3-ol 2,4-heptadienal and pentadecane have been characterised as important volatile

compounds contributing to the formation of off-flavor in LC-PUFA containing oils (Benedetti and Mannino, 2009, Jacobsen C., 2010, Maire et al., 2013). Variations of the volatiles are described in detail in paper(5). So that here the variations in two compound are described. The contents of 1-penten-3-one were mainly influenced by the storage time (P value <0.05). During the first days of storage, the amounts of 1-penten-3-one rapidly increased, reached a plateau at day 14 and then decreased, with the same pattern for samples stored at different temperatures. The highest amount was observed for the samples stored at 40°C at day 14, which was significantly greater than 5°C stored samples. Effect of packaging conditions was not significant, excluding the samples stored at 5°C (day 56). It has been reported that 1-pentene-3-ol (**Table 6.2**), which is derived from the oxidation pathway of EPA further oxidise onto 1-pentene-3-one (Venkateshwarlu et al., 2004).

Table 6.2. Contents of 1-penten-3-ol and 1-pentene-3-one (ng/g of DW sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q).P < 0.05.

1-penten-3-ol

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	3992±342,3	b,x	3992±342,3	a,x	3992±342,3	a,x
	AMB	8479±567,6	d,x,p	7214±1179	b,x,p	10593±357,2	d,y,p
14	VAC	8162±595,5	d,x,p	12699±1317	d,y,q	11619±3217	d,y,p
	AMB	4479±655,5	bc,x,p	4845±648,8	a,x,p	9715±1294	c,y,p
28	VAC	4526±1018	bc,x,p	9426±3747	c,y,q	10523±338,2	d,y,q
	AMB	5527±271,8	c,x,p	8958±203,6	bc,y,q	4118±507,7	ab,x,p
42	VAC	8892±1910	d,y,q	3187±196,1	a,x,p	9280±1299	c,y,q
	AMB	3570±445,5	a,x,q	3023±64,05	a,x,p	5415±1268	b,y,p
56	VAC	2053±433,3	a,x,p	4135±248,9	a,xy,p	5133±1251	b,y,p

1-penten-3-one

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	1902±238.2	a,x	1902±238.2	a,x	1902±238.2	a,x
	AMB	8413±312.5	e,z,p	5450±1319	d,y,p	3798±383.1	c,x,q
14	VAC	8368±773.9	e,z,p	4888±900.7	c,y,p	2370±1707	a,x,p
	AMB	4013±1008	c,x,p	4465±891.9	c,x,p	3932±2100	c,x,q
28	VAC	5463±1881	d,y,q	4793±1500	c,y,p	2173±251.8	a,x,p
	AMB	5172±583.9	d,y,q	2707±980.9	a,x,p	6223±1623	d,z,q
42	VAC	4496±1100	c,y,p	2687±349.4	b,x,p	3383±646.5	b,y,p
	AMB	3232±448.8	b,x,p	2848±250.5	b,x,p	3082±969.1	b,xq
56	VAC	2764±782.1	a,y,p	2633±81.16	a,xy,p	2000±505.3	a,x,p

The higher amounts of 1-penten-3-ol and 1-penten-3-one in samples stored under vacuum compared to ambient pressure was also reported by Benedetti and Mannino (2009). On the other hand, the presence of ketones such as 1-penten-3-one, 3-pentanone, and alcohols such as 1-penten-3-ol, in various strains of microalgae including *Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis* sp., *Nannochloropsis oculata*, and *Chlorella vulgaris* was reported by Van Durme et al. (2013). Considering the absence of EPA in the later species (*Chlorella vulgaris)* it can be concluded that other chemical or metabolically reactions may be involved in the production of these compounds. The contribution of 1-octen-3-ol to the formation of fish-like aroma was reported by Van Durme et al. 2013.

6.4. Conclusion

During dry storage of microalgae *Nannochloropsis salina* biomass, the storage time and temperature strongly influenced the oxidation reactions, which results in deterioration of bioactive compounds such as carotenoids, tocopherols and LC PUFA and HUFA. In a freeze-dried microalgae biomass, the lipid deterioration occurred both due to enzyme-induced lipolysis and autoxidation. The contents of carotenoids and α-tocopherol which are known as natural antioxidants, decreased during the storage. However, these natural antioxidants probably prevented/retarded oxidative deterioration of EPA. Oxidation reactions, which resulted in the creation of primary and secondary products, occurred mainly during the first days of storage. The volatile compounds declined further due to the formation of more stable compounds, e.g. by the bonding to some amino acids or decomposition to tertiary oxidation compounds. Storage of freeze dried microalgae at a low temperature (e.g. 5℃) is more effective than oxygen-reduced storage conditions such as vacuum packaging. Further investigations are required to find additional methods for extending the shelf life of dried microalgae.

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Chapter 7; Conclusions

This chapter summarises the main findings of the project in relation to the hypotheses and corresponding objectives described in Chapter 1.

7.1. Screening of microalgae species based on their ability to grow on ICW and the biochemical composition

The industrial process water (ICW) represented an excellent source of nutrients such as nitrogen and phosphorous. Various microalgae species including *Chlorella pyrenoidosa, Chlorella vulgaris, Desmodesmus* sp., *Chlorella minutissima*, and *Chlorella sorokiniana*, all from Chlorophyte, grew on ICW as the main and unique nutrient source.

The microalgae *Chlorella pyrenoidosa* was found as the best candidate, due to the ability to grow on 100% industrial process water (ICW), and yielded reasonable amounts of biomass in laboratory scale (1.84 g/L). The biomass of ICW grown *Chlorella pyrenoidosa* presented extremely high levels of protein (58.3± 3.2 % DW), promising amino acid composition and-and high content of carotenoids (6.72± 0.21 mg/g DW) but was lacking LC-PUFA. Protein and pigment contents were enhanced by higher percentages of ICW in the growth media. Besides this, the effective valorization of nutrients which reduces the biological oxygen demand of the ICW is a significant benefit as it saves the money which should be paid for waste water treatment, before releasing it to the environment.

Cultivation of *Nannochloropsis salina* on a mixture of industrial process water and F/2 standard growth media enhanced the nutritional quality of *Nannochloropsis salina* when compared to algae cultivated in the standard F/2 medium. The resulting biomass was a rich source of EPA (37.7±0.77 % of total fatty acids in laboratory scale experiments) and also a good source of protein (amino acids), tocopherols and carotenoids for potential use in aquaculture feed industry.

7.2. Evaluation of the effects of growth medium and cultivation duration on the chemical composition of microalgal biomass

During the cultivation, the protein content declined for nearly all microalgae species, while total lipids, EPA (for *Nannochloropsis salina* and *Nannochloropsis limnetica*) and natural antioxidants such as tocopherol and carotenoids increased. The cultivation duration did not influence the amino acid compositions. Microalgae from the class chlorophtye grew well on ICW as the main, unique nutrient source and produce promising amounts of biomass. For eustigmatophytes and cyanobacteria, partial substitution of standard growth medium with ICW to levels less than 25% has shown no adverse effect (eustigmatophytes) or even improved the growth (cyanobacteria) compared to the algae cultivated on standard growth media as a reference.

7.3. Evaluation of the effect of large-scale cultivation on the chemical composition and growth of selected species.

Large-scale cultivation of *Nannochloropsis salina* in a flat panel photobioreactor confirmed the laboratory-scale findings. The algae growth rate at winter condition of Denmark was slow, but the nutritional properties such as such as EPA (44.2±2.3 % total fatty acids), α-tocopherol (431±28 μg/g of biomass dry weight), protein and carotenoids improved compared to laboratory scale cultivated microalgae. The microalgae *Chlorella pyrenoidosa* was also grown in a tubular photobioreactor during a short period. Results of the short term large scale experiment also confirmed the laboratory results such as compatibility of the algae to 100% ICW and high contents of biomass protein (58.3±1.0% DW). However, the biomass from large scale cultivated *chlorella pyrenoidosa* reached 6 g/L, which was much higher than laboratory scale results (1.8 g/L).

7.4. Development and testing of the strategies for the quality control of the algae biomass during the cultivation, processing and the storage.

The combination of pigment and fatty acid analyses was proven to be an effective and fast tool for characterising algal groups in both pre and post-harvest applications. Both the HPLC based method for the analysis of pigments and the two step direct trans esterification method for the analysis of fatty acids (DT) which were developed in this project, have been extensively used for the control of the culture purity at both laboratory and large scale experiments. Besides this DT method represents a rapid, routine and reliable control measure to verify the quality and/ or purity of microalgae biomass during cultivation or application as a food/feed ingredient. The combination of fatty acid and pigment composition provides very useful data which might be used as a tool for the investigation of the purity or deteriorations of the microalgae biomass.

7.5. Optimum storage condition for microalgae biomass as a fish feed ingredient.

During dry storage of microalgae *Nannochloropsis salina* biomass, the storage time and temperature strongly influenced the oxidation reactions, which results in deterioration of bioactive compounds such as carotenoids, tocopherols and LC PUFA and HUFA. In a freeze-dried microalgae biomass, the lipid deterioration occurred both due to enzyme-induced lipolysis and autoxidation. Carotenoids and α-tocopherol which are known as natural antioxidants decompose during the storage. However, these natural antioxidants probably prevented/retarded oxidative deterioration of EPA. Oxidation reactions, which resulted in the creation of primary and secondary products, occurred mainly during the first days of storage. The volatile compounds declined further due to the formation of more stable compounds, e.g. by the bonding to some amino acids or decomposition to tertiary oxidation compounds. Storage of freeze-dried microalgae at a low temperature (e.g. 5℃) is more effective than oxygen-reduced storage conditions such as vacuum packaging. Further investigations are required to find additional methods for extending the shelf life of dried microalgae.

7.6. Development and testing a specific downstream processing for the production of microalgae biomass as a fish fed ingredient.

Aiming at the utilisation of microalgae as a feed ingredient, the processing set up shall be feasible and without toxic chemicals as a process aid, while reasonably preserving valuable ingredients such as essential amino and fatty acids, pigments, and proteins. Harvest of microalgae by membrane microfiltration and subsequent up-concentration using an efficient and feasible centrifuge provides a biomass paste which includes nearly all of the bioactive compounds. The process developed in this project included cross flow microfiltration by SiC (0.1μm) ceramic membranes, up concentration by bowl centrifuge at 6500±500 g and finally drying by the swirl (spin) flash dryer, which was designed specifically and tested on microalgae samples. Energy consumption per kg of the product was evaluated as 2.2 KWh, which is comparatively lower than common commercial method (spray drying). It could also be pointed out that the deterioration of value-added bioactive compounds such a carotenoids is lower in the invented method compared to spray drying.

From an economical point of view, swirl (spin) flash dryer uses 28% less energy per kg weight of dried product, require less investment and even less area for the installation and application, when compared to spray drying in the same condition. The dryer design is complex, but the construction does not require special high tech units such as atomiser in a spray dryer. On the other hand, this system could be operated in small scales which is desired for the microalgae cultivation systems, which produce between 10-100 kg of the paste per day. This system can handle the high viscosity microalgae paste in which the main part of the water is removed using mechanical methods, including microfiltration and centrifugation. So that less heat energy is required for the drying which results in lower degradation of the valuable compound in the microalgae biomass. The prototype swirl flash dryer was designed for the specific application of microalgae drying, but continual improvement would always be required. The system has only been tested on a biomass from *Nannochloropsis salina.* Drying efficiency of the prototype dryer should be tested on various samples of microalge species from different classes and with diverse physiochemical properties. These drying trials result in the improvements/ modification of feeding unit, drying tyube(length, baffles, etc.), air inlet(numbers, dimensions and arrangement) and scrapping paddles.

Chapter 8; Future perspectives

Microalgae represent a promising and rich source of bioactive compounds, which can fulfil the future requirements of bioactive compounds as a feed, food or pharmaceutical ingredient. Microalgae production is a green, sustainable and environment-friendly business which uses less land and water compared to commercial crops such as soybean and corn, while yields more oil and protein. This study investigated various aspects of the production, processing, analysis and storage of the microalgae as a fish meal ingredient and showed how carefully selected microalgae could grow on industrial process water and convert nutrients to biomass with desired chemical composition. The study also revealed how processing and storage conditions could influence the chemical composition of microalgae biomass. The following items represent the future perspectives of the application of microalgae as a fish feed ingredient.

8.1. Selection, evaluation and testing of new microalgae species

Among more than 200,000 microalgae, few of them are well studied. Further investigations are critically required to find/identify algal species which can produce bioactive compounds. These new species shall also be evaluated by chemical and biological methods to ensure that their biomass does not contain substances which may have an adverse effect on the aquatic animals.

8.2. Analytical methods and quality criteria

Microalgae are a broad group of organism, industrialisation of algal products requires intensive knowledge for the identification and quantification of the biochemical composition of microalgae biomass. Besides this, the setting of criteria for quality control and monitoring of the purity of culture is critically required. These criteria when globally accepted can improve the traceability of the product and reduce the risk of contamination with either poisonous or unknown algae species or even adulteration with cheaper products. The majority of the methods which are currently used for the analysis of microalgae bio composition are non-specific and not validated for microalgae biomass. Analytical methods which are applied to microalgae might be discussed, improved, developed and validated by technical working group in organizations such as Codex Alimentarius, International Standardization Organization (ISO).

8.3. Large scale cultivation

Commercialization of the algal biomass requires solutions to reduce the cultivation and production costs. Cultivation of microalgae on a cheap source of nutrients reduces the cultivation cost so that the product can compete for other sources which are less sustainable. Utilisation of various types of wastes is a possible opportunity when they do not contain any harmful substances. Effluents from anaerobic digestion of wastes from known sources (e.g. from food factories) can be evaluated for this

reason. The bio utilisation of the effluent reduces the cultivation costs by deduction of the price of growth medium. This process also benefits from valorization of the effluent which reduces the environmental polluting load caused by nutrients and thus saves the money which the factory should normally pay for treating the effluent before it can return to the environment. On the other hand, improving the efficiency of light, nutrients and CO_2 utilisation by microalgae results in higher productivity of biomass and target compounds. More research in laboratory and large scale is required for developing bioreactors which are able to overcome the limitations of the currently developed photo bioreactors. However achieving this requires detail knowledge in various aspects such as; efficient and selective light distribution, physio chemical properties of algae species such as maximum tolerated static pressure, cleaning, monitoring and control techniques.

8.4. Harvest, up-concentration and drying of microalgae

Commercial production of microalgae as aquaculture feed is still expensive and unclear in some technical aspects. The numerous challenges in algae production and use as a fish feed extend across the entire process chain, including the selection of suitable algal species, the harvest of the biomass from the suspension, and processing (concentration, drying) to form a meal containing essential nutritional compounds. Downstream processing is also required to extend shelf-life of the product. This study introduced a novel drying concept which individually designed for microalgae. However, the dryer unit was tested by a limited number of species, so that further studies require extending the scope of the system. On the other hand, a prototype system could be improved trough further drying experiments

8.5. Application of microalgae biomass and its derivatives in food and feed

Microalgae biomass may include various valuable compounds which are not either of interest for fish meal or being deteriorated during the processing and storage. A practical example is Lutein which was produced in extremely high concentrations by ICW grown *Chlorella vulgaris*. Lutein is not desired in fish meal industry as it results in the development of yellow colour in fish meat. However, lutein is heat sensitive carotenoid, so that extraction of lutein as a byproduct and by non-chemical methods such as super critical CO_2 extraction, reduces the final price of the biomass, which still contains desired compounds such as amino acids. Application of modern technologies for fractionation and selective extraction of bioactive compounds results in production of various products such as pigments, proteins, and LC PUFA rich lipids which can be sold separately. This strategy can reduce the final price of each product.

Paper 1

Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater

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Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater

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Abstract: This study aimed at investigating the potential of microalgae species grown on industrial waste water as a new source of natural antioxidants. Six microalgae from different classes, including *Phaeodactylum* sp. (Bacillariophyceae), *Nannochloropsis* sp. (Eustigmatophyceae), *Chlorella* sp., *Dunaniella* sp., and *Desmodesmus* sp. (Chlorophyta), were screened for their antioxidant properties using different *in vitro* assays. Natural antioxidants, including pigments, phenolics, and tocopherols, were measured in methanolic extracts of microalgae biomass. Highest and lowest concentrations of pigments, phenolic compounds, and tocopherols were found in *Desmodesmus* sp. and *Phaeodactylum tricornuotom* microalgae species, respectively. The results of each assay were correlated to the content of natural antioxidants in microalgae biomass. Phenolic compounds were found as major contributors to the antioxidant activity in all antioxidant tests while carotenoids were found to contribute to the 1,1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging activity, ferrous reduction power (FRAP), and ABTS-radical scavenging capacity activity. *Desmodesmus* sp. biomass represented a potentially rich source of natural antioxidants, such as carotenoids (lutein), tocopherols, and phenolic compounds when cultivated on industrial waste water as the main nutrient source.

Keywords: microalgae; phenolic compounds; antioxidants; wastewater; carotenoids; tocopherols; DPPH; FRAP

1. Introduction

Algae are one of the oldest living organisms of planet earth. Microalgae can grow in quite different environments, like sea, and desert [1]. In recent years algae have been in the center of interest as a sustainable, rich source of bioactive compounds, like phenolic compounds, fatty acids, amino acids, and carotenoids. There has also been a global trend to replace artificial antioxidants with natural antioxidants during the past two decades. Antioxidants are increasingly being used in food supplements as bioactive compounds and in functional foods to increase their shelf life and prevent unwanted lipid oxidation. Nearly all commercially available natural antioxidants are derived from terrestrial plants [2]. It is, however, believed that microalgae could be an alternative resource of natural antioxidants as they are much more diverse than other sources like plants [3]. The global market for micro-algae-based food and feed supplements/nutraceuticals is well developed

and with a great potential for growth, so investigation of antioxidative properties and natural antioxidant composition of microalgae biomass is important. There are a number of reports on the evaluation of antioxidant activity of some microalgae and cyanobacteria species belonging to the genera of *Botryococcus* [4], *Chlorella* [5–7], *Dunaliella* [8], *Nostoc* [9], *Phaeodactylum* [10], *Spirulina* [11,12], *Nannochloropsis*, *Chaetoceros* [13], *Halochlorococcum*, *Oltamannsiellopsis* [14], and *Navicula clavata* [7].

Carotenoids are a family of yellow to orange-red terpenoid pigments synthesized by photosynthetic organisms as well as some bacteria and fungi [15]. Carotenoids can act as antioxidants by scavenging and deactivating free radicals [16]. Carotenoids include two classes; xanthophylls, which contain oxygen, and carotenes, which are purely hydrocarbons and contain no oxygen. All xanthophylls synthesized by higher plants e.g., violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and lutein, can also be synthesized by green microalgae; however, these possess additional xanthophylls, e.g., loroxanthin, astaxanthin, and canthaxanthin. Diatoxanthin, diadinoxanthin, and fucoxanthin can also be produced by brown algae or diatoms [15]. Several studies have shown that carotenoids contribute significantly to the total antioxidant capacity of microalgae [16–18].

The term "polyphenol" includes more than 8000 compounds with great diversity in structure. They can be divided into 10 different classes depending on their basic chemical structure [19]. Phenolic compounds are recognized as important natural antioxidants. Polyphenols act as antioxidant through single electron transfer and through hydrogen atom transfer [16]. Some studies suggest that the content of phenolic substances in microalgae is lower than or equal to the minimum amounts reported for terrestrial plants [4], and just include phenolic acids. However some recent studies showed that several classes of flavonoids, such as isoflavones, flavanones, flavonols, and dihydrochalcones can also be found in microalgae [18]. This clearly demonstrates that microalgae are able to produce also more complex phenolic compounds, so characterization and identification of phenolic compounds in microalgae are required, especially as they may contain novel phenolic compounds [19].

There are only few published studies regarding the identification and quantification of phenolic composition in microalgae species [8,11,20,21]. Abd El-Baky *et al*. [20] found phenolic compounds including gallate, chlorogenate, cinnamate, pinostrobate, and *p*-OH-benzoates in *Spirulina* sp. Other researchers reported salicylic, *trans*-cinnamic, synapic, chlorogenic, and caffeic acids as the main phenolic acids in this microalgae species [12]. The results of these studies show the intensive effects of growth media on the phenolic composition of microalgae species. In a very recent UPLC-MS/MS study, simple phenolics and hydroxycinnamic acids (ferulic acid and *p*-coumaric acid) were detected in *Chlorella vulgaris*, *Haematococcus pluvialis*, *Diacronema lutheri*, *Phaeodactylum tricornutum*, *Tetraselmis suecica*, and *Porphyridium purpureum* microalgae species [22].

There are many studies concerning the screening of microalgae species based on their antioxidative properties by using different *in vivo* and *in vitro* assays. Goiris *et al.* [16] screened 32 microalgal biomass samples for their antioxidant capacity using three antioxidant assays, and both total phenolic content and carotenoid content were measured. The study revealed that industrially-cultivated samples of *Tetraselmis suecica*, *Botryococcus braunii*, *Neochloris oleoabundans*, *Isochrysis* sp., *Chlorella vulgaris*, and *Phaeodactylum tricornutum* possessed the highest antioxidant capacities and, thus, could be potential new sources of natural antioxidants. The results also showed that both phenolic and carotenoids contributed significantly to the antioxidant capacity of microalgae.

The main pollutants in different wastewater sources are nitrogen (N) and phosphorus (P) in different forms, which on the other hand, are necessary nutrients for algae growth. Recent studies have shown that some microalgae can grow on wastewater, uptake the nutrients such as N and P, reduce the biological oxygen demand (BOD) and produce biomass, which can be used for different purposes [23]. Wastewater can provide water medium as well as nearly all necessary nutrients for cultivation of microalgae. Combination of wastewater treatment and algae cultivation could be a feasible, environmentally-friendly approach for sustainable production of algae-based bioactive compounds [23]. The bio-refinery approach consists of sustainable production of biomass through

an integrated process. As an example, use of these strategies may offer an inexpensive alternative to the conventional technological routes of production of natural pigments [24].

The aim of this study was to investigate natural antioxidant composition and antioxidative properties of some microalgae species from different classes including *Phaeodactylum* sp. (Bacillariophyceae), *Nannochloropsis* sp. (Eustigmatophyceae), *Chlorella* sp., *Dunaniella*, and *Desmodesmus* (Chlorophyta) which were grown autotrophically on industrial waste water.

2. Results and Discussion

2.1. Extraction of Phenolics and Carotenoids

In microalgae, carotenoids and phenolic compounds are surrounded by cell wall and, therefore, procedures that can break down cell walls with minimum risk of damage are needed. An efficient extraction requires that the solvent penetrates into the cell and dissolves the target compounds corresponding to the polarity. Many different non-conventional extraction methods including electrical pulsed electric fields (PEF), high-voltage electrical discharges (HVED), high-pressure homogenization, ultrasounds, microwaves, sub- and supercritical fluid extraction, have been proposed as suitable techniques to achieve this purpose [25]. Combination of these techniques with the solvent extraction increase the yield of extraction. In a recent study, a high level of extraction of pigments and other bioactive compounds is reported by using the combination of pulsed electric field assisted extraction and solvent extraction in biomass of *Nannochloropsis* spp. [26]. Ultrasound-assisted solvent extraction is reported as a promising tool to recover high-added value compounds from the microalgae *Nannochloropsis* spp. [27]. Low temperature sonication could enhance the cell rupture efficiency, without negative mechanical or heat induced effects on sensitive carotenoids. In this study, we evaluated the combination of sonication technique with four common extraction solvents for the extraction of both carotenoids and phenolics in samples of *Chlorella sorokiniana* by procedures described at Sections 3.1 and 3.2 of this paper. In the ultrasonic process, the microalgal cells are disrupted by shock waves from cavitation bubbles, enhancing the liberation of valuable compounds. The sonication process utilizes the cavitation to disrupt the cell wall and results in the physical effects of micro-turbulence and velocity/pressure shockwaves. Micro-turbulence provides intense mixing, while shockwaves cause disruption of the cell wall [25]. Methanol extraction showed the highest concentration of both carotenoids and phenolics (Figure 1a). For all of the pigments higher levels of extraction were achieved by using methanol (Figure 1b), even for beta carotene, which as a hydrocarbon lacking functional groups, is very lipophilic and more soluble in acetone [28].

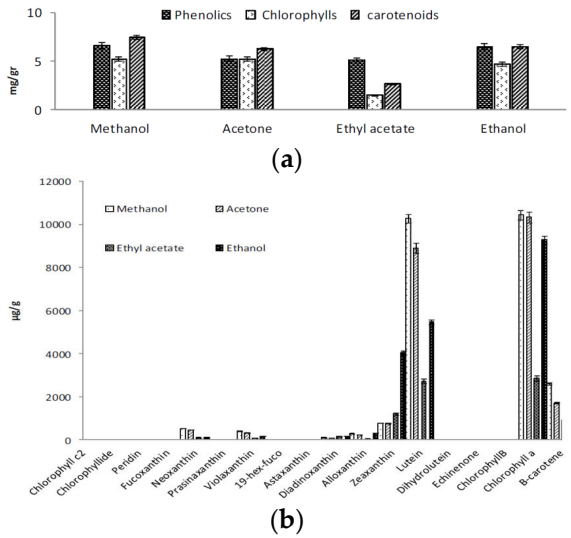

Figure 1. (**a**) Evaluation of extraction yield of different solvents for phenolic compounds and pigments; and (**b**) evaluation of effects of different solvents on carotenoids content and composition.

Methanol is one the most favored solvents which is used for the extraction of polar compounds such as phenolic compounds and flavonoids. It has previously been shown that methanol extract of microalgae has more antioxidative power compared to extracts obtained with other common solvents [6,7,29] and it was also claimed that methanol can disintegrate cell membranes more than other solvents [30]. In the present study pigments were extracted from dried samples, which prevent the risk of degradation of pigments. Thus, development of chlorophyll derivatives (e.g., pheopigment) arising normally from sample processing was reduced or prevented.

2.2. Total Phenolics, Flavonoids, and Phenolic Composition

The phenolic contents based on Folin method varied from 7.72 ± 0.08 to 3.16 ± 0.04 (mg/g GAE) with statistically significant differences among the species (Table 1). The highest and lowest concentrations were found in *Desmodesmus* (De.S) and P.T respectively. There were no significant differences between C.S1 and C.S2, so light intensity did not show significant effect on the phenolic content in *Chlorella sorokinia* species (C.S1 and C.S2). Comparison of the results by simple regression shows a statistically significant relationship between phenolic composition by HPLC and total phenolic compounds at the 95.0% confidence level. The correlation coefficient equals 0.88 which indicates a strong relationship between the results, indicating that identified phenolic acids are the main contributors to the compounds measured by the Folin method. Our result falls within the range given by previous reports [3,7,9,12,27,31–35]. Hajimahmoodi *et al.* [33] reported that *Chorella vulgaris* had the highest total phenolic content among samples of water extracts from 12 strains of microalgae. The study also showed that phenolic compounds were major contributors to the microalgae antioxidant capacity. Production of phenolics as well as other antioxidant compounds in microalgae depends on the growth conditions and stresses such as oxidative stress, so it shall be considered when the results are being compared to other studies.

Table 1. Total phenolics, flavonoids, carotenoids, and tocopherols in microalgae biomass.

Species	Total Phenolics (mg/g) *	Total Tocopherols (μg/g)	Total Carotenoids (mg/g)	Total Flavonoids (mg/g) **
De.S	7.72 ± 0.08 a	361.9 ± 23 a	6.70 ± 0.01 a	4.03 ± 1.10 a
Du.S	4.52 ± 0.05 d	125.2 ± 23.5 b	4.83 ± 0.01 d	3.61 ± 1.09 a
N.L	5.78 ± 0.12 c	21.18 ± 0.05 d,e	2.56 ± 0.02 g,f	2.6 ± 0.30 a,b
P.T	3.16 ± 0.04 f	13.12 ± 0.01 e	4.60 ± 0.03 e	0.84 ± 0.12 a
N.S	6.45 ± 0.25 b	44.08 ± 3.11 c	5.296 ± 0.01 b	3.18 ± 0.59 a
C.S1	5.86 ± 0.06 c	34.13 ± 0.37 c,d	4.978 ± 0.06 c	2.49 ± 0.7 a,b
C.S2	5.76 ± 0.12 c	33.74 ± 0.27 c,d,e	2.92 ± 0.15 f	2.41 ± 0.9 a,b

Values are given as mean (n = 3) ± standard deviation (absolute value). For each column, same letters indicate similar values ($p < 0.05$); * As gallic acid equivalent; ** As quercetine equivalent.

Our results for identified phenolic (HPLC) varied from 10.07 ± 0.04 μg/g for N.S to 5.10 ± 0.12 μg/g for P.T. as shown in Table 2. Only simple phenolic acids were identified by using reference standards, so the results did not include more complex phenolics. Identified phenolic acids include gallic acid, 2,5-dihydroxy benzoic acid, 3,4-dihydroxy benzoic acid, caffeic acid, ferulic acid, *p*-coumaric acid, salycilic acid, and cinnamic acid (Table 2). In this study we detected hydroxy cinnamic acids in green algae including De.S, Du.S, C.S1, and C.S2, which belong to the same class. All samples excluding P.T contain ferulic acid while 3, 4 dihydroxy benzoic acid was only found in PT and N.S., and *p*-coumaric acid was identified in all samples excluding N.L. Two batches of *Chlorella sorokiniana* grown in different light intensities had the same phenolic acid profile, but the total identified phenolic acids was slightly higher in the sample grown in normal light intensity (C.S2).Characterization of phenolic acids in microalgae species has been carried out in other studies, which can confirm some of the results of this study. One study reported the presence of highly-polar phenolic compounds of C6-C11 or C6 phenolic skeletons [11],

single phenols including protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, caffeic, chlorogenic acid, 4-hydroxybenzaldehyde, and 4-dihydroxybenzaldehyde in *Spirulina* [20], hydroxycinnamic acids (ferulic acid, *p*-coumaric acid) in *Chlorella vulgaris*, *Haematococcus pluvialis*, *Diacronema lutheri, Phaeodactylum* sp., *Tetraselmis suecica*, and *Porphyridium purpureum*, and *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, caffeic, and chlorogenic acid; 4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde in *Spongiochloris spongiosa* and *Spirulina platensis, Anabaena doliolum, Nostoc* sp., and *Cylindrospermum* sp. [22].

Table 2. Identified phenolic composition of microalgae biomass.

Phenolic Compounds (μg/g)	De.S	Du.S	N.L	P.T	N.S	C.S1	C.S2
Gallic acid	4.32 ± 0.01	nd	2.30 ± 0.02	nd	2.75 ± 0.03	nd	nd
2,5 dihydroxy benzoic acid	nd	nd	nd	nd	nd	nd	nd
3,4 dihydroxy benzoic acid	nd	nd	nd	1.64 ± 0.02	2.90 ± 0.07	nd	nd
Caffeic acid	1.11 ± 0.01	1.34 ± 0.04	1.37 ± 0.04	nd	nd	3.81 ± 0.03	3.12 ± 0.20
Ferulic acid	1.41 ± 0.04	4.07 ± 0.03	2.45 ± 0.04	nd	2.90 ± 0.05	2.81 ± 0.03	2.80 ± 0.20
p-Coumaric acid	1.91 ± 0.01	0.67 ± 0.02	nd	1.56 ± 0.12	0.29 ± 0.09	1.97 ± 0.05	1.16 ± 0.09
Salycilic acid	nd	nd	0.55 ± 0.07	1.91 ± 0.20	1.32 ± 0.01	nd	nd
Cinnamic acid	0.64 ± 0.01	nd	0.92 ± 0.01	nd	nd	0.47 ± 0.02	0.13 ± 0.04
Total	9.40 ± 0.09 b	6.09 ± 0.04 f	7.60 ± 0.05 d	5.10 ± 0.12 g	10.07 ± 0.03 a	9.06 ± 0.09 c	7.26 ± 0.08 e

Values are given as mean (n = 2) ± standard deviation (absolute value). Same letters indicate similar values ($p < 0.05$). nd = Not detected.

As shown in Figure 2A there are other unidentified compounds, which might stem from flavonoids with complex structure. It has been shown that coumaric acid, which is the precursor of the flavonoid synthesis, is present in microalgae species. It has also been shown that the metabolic capacity for production of flavonoids is present in all major evolutionary lineages of microalgae and cyanobacteria [22]. Concentration and composition of the phenolic compounds in microalgae biomass could be affected by both species and growth conditions. Spectrophotometric assay confirmed the presence of flavonoids in the microalgae species grown on industrial wastewater (Table 1). Evaluation of the relationship between total phenolic compounds and total flavonoids indicates that the correlation based on R^2 just explains 11.7% of the variability in total phenolic compounds, demonstrating a weak relationship between total phenolic and flavonoids. The reason might be that the Folin reagent does not only measure phenols, and can generally react with other reducing substances. It, therefore, measures the total reducing capacity of a sample, including some nitrogen-containing compounds, metal complexes, vitamin derivatives, and organic acids [36]. While spectrophotometric analysis of flavonoids is a more specific method. Furthermore in a complex sample, the interference from other compounds, e.g., pigments can affect the precision of the result of spectrophotometric analysis of flavonoids.

2.3. Total Tocopherols

Total tocopherol content in methanolic extracts varied highly between the species (Table 1), with highest amount in De.S (361.9 ± 23.1 μg/g) and lowest in P.T (13.12 ± 0.01 μg/g). There was no statistically significant difference between C.S1 and C.S2 so the light intensity did not significantly affect the amounts of tocopherols in these samples. Tocopherol composition mostly includes α-tocopherol (Figure 2B) in all samples. As shown in Table 1, total tocopherol content was higher in some green algae (Chlorophyceae) compared to others (Eustigmatophyceae and diatoms). Only few publications reported the tocopherol composition of microalgae. The effects of nitrogen source, concentration, and growth phase on tocopherol concentration in *Nannochloropsis occulata* was reported by Durmaz *et al.* [37]. In this research high amount of α tocopherol was reported as 2326 ± 39 μg/g DW for this specie. Other studies reported total tocopherols as 283.6 μg/g, 153.2 μg/g, and 157.7 μg/g for *E. gracilis, Dunaliella salina*, and *Tetraselmis suecica,* respectively [38], and 421.8 μg/g, 58.2 μg/g, 116.3 μg/g, and 669.0 μg/g of α-tocopherol for *Tetraselmis suecica, Isochrysis galbana, Dunaniella tertiolecta*, and *Chlorella stigmatophora*, respectively [39]. Our results

for De.S are comparable to these reports showing the potential of the this specie for production of tocopherol when cultivated on industrial wastewater.

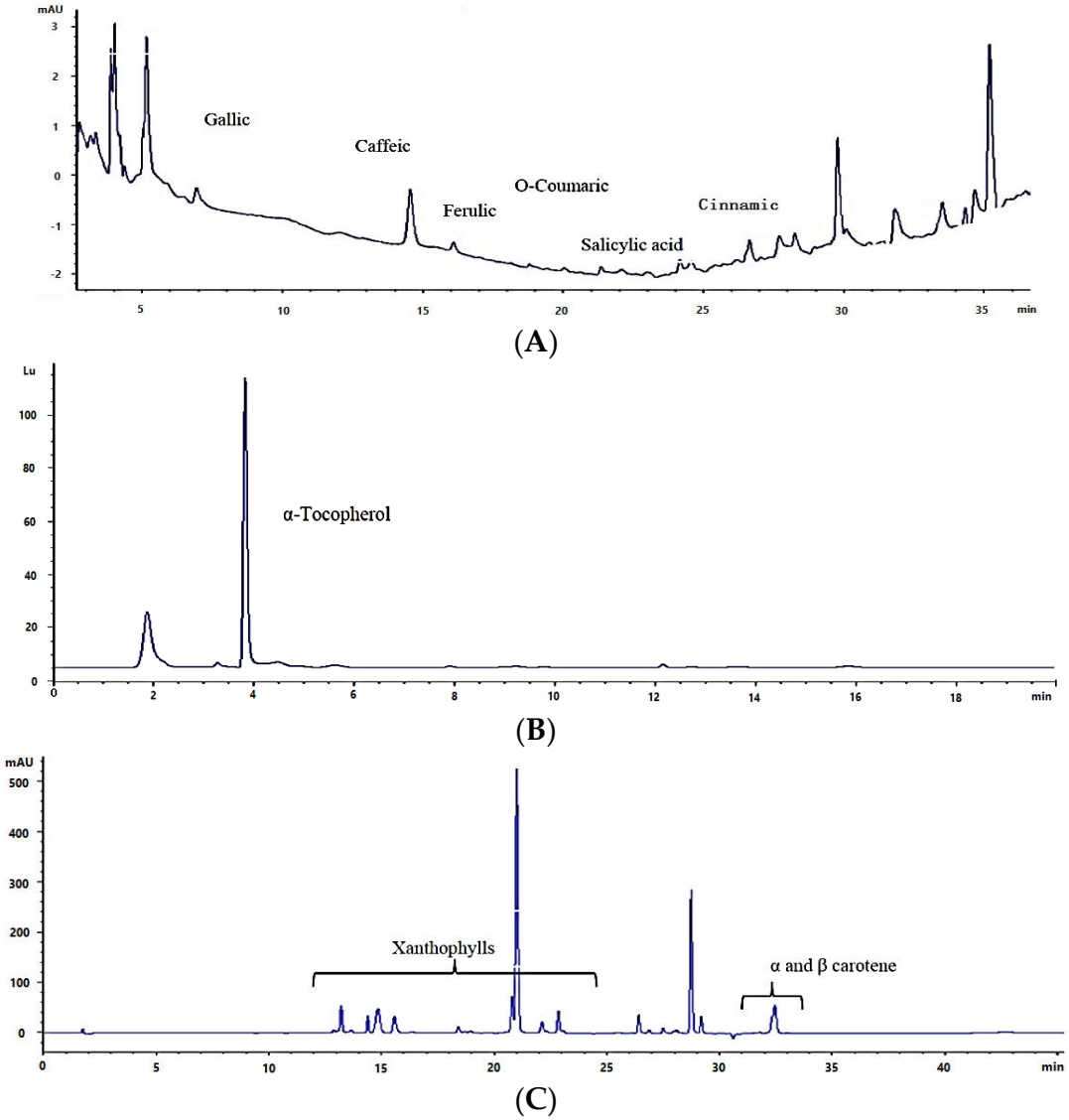

Figure 2. HPLC chromatograms of (**A**) phenolic compounds; and (**B**) tocopherols and (**C**) carotenoids.

2.4. Total Carotenoids and Carotenoid Composition

Total carotenoids varied highly between species (Table 1). Highest amounts of total carotenoids were detected for De.S and lowest for N.L, at 6.70 ± 0.017 mg/g, and 2.56 ± 0.02 mg/g, respectively. Light intensity had statistically significant effect on the amounts of carotenoids in *Chlorella sorokiniana*. Thus, when exposed to high light intensity carotenoid production increased to 4.98 ± 0.07 mg/g in C.S1, compared to C.S2 with 2.92 ± 0.15 mg/g. Carotenoids accumulation can be affected by growth conditions e.g., light intensity and growth media composition, so again it can be considered that compatibility of *Desmodesmus* sp. to the growth conditions resulted in higher accumulation of carotenoids. Total carotenoid content in the biomass falls within the range given previously by several reports [15,16,40–42]. It has been shown that both carotenoid content and composition can be influenced by culture conditions such as growth media composition [16]. We used industrial wastewater as a source of nutrients, so comparison of the results with other studies shall be done with this consideration.

Carotenoid compositions of the strains also varied between different classes (Table 3).The main carotenoid compound in samples from green algae class was lutein with the amounts 5111 μg/g, 3014 μg/g, 3220 μg/g, and 2069 μg/g for De.S, Du.S, C.S1, and C.S2, respectively. In both samples N.S and N.L, which belong to Eustigmatophyceae class, the main carotenoid was violaxanthin at 1679 μg/g in N.S and 1228 μg/g in N.L. In diatom P.T the most abundant carotenoids were diadinoxanthin and diatoxanthin as 2166 μg/g and 1558 μg/g, respectively. The highest accumulation of β-carotene was detected in N.S, followed by C.S1 and Du.S as 2223 μg/g, 1039 μg/g, and 743 μg/g, respectively. *Dunaniella salina* is famous for its ability to accumulate β-carotene [11], but our results do not confirm that. The fact that production of β-carotene can be highly affected by suboptimal growth conditions such as growth media composition, starvation, and light intensity was shown in different studies [28] and could explain why Du.S did not accumulate high levels of β-carotene. Astaxanthin, antheraxanthin, 19-butyro-fucoxanthin, and cantaxanthin were detected just in C.S1, Du.S, P.T, and N.S, respectively. It has been shown previously that lutein and β-carotene prevailed among carotenoids in *Desmodesmus* microalgae followed by xanthophylls of the violaxanthin cycle (violaxanthin, antheraxan, zeaxanthin) and neoxanthin that are typical of Chlorophyta family [34].

Table 3. Pigments composition of microalgae biomass. Amounts are represented in (μg/g) unidentified peaks are less than 5% of total pigments for all samples.

Pigments (μg/g)	De.S	Du.S	N.L	P.T	N.S	C.S1	C.S2
Chlorophyll c3	nd	nd	nd	nd	nd	nd	nd
Unknown	212.2 ± 5	351.0 ± 7.5	nd	nd	nd	269.7 ± 4.6	80.78 ± 1.3
Chlorophyllide	nd	nd	nd	nd	nd	nd	nd
Peridin	nd	nd	nd	nd	nd	16.70 ± 0.1	4.69 ± 0.1
Vaucheriaxanthin	nd	nd	164.8 ± 2.6	nd	85.16 ± 0.6	nd	nd
19-But-Fucoxanthin	nd	nd	nd	50.75 ± 2.2	nd	nd	nd
Fucoxanthin	nd	nd	183.2 ± 9.8	264.5 ± 29	13.05 ± 0.1	104.8 ± 5.5	22.2 ± 0.2
Neoxanthin	158.3 ± 2.5	103.2 ± 12	423.4 ± 28	nd	53.45 ± 3.2	48.29 ± 1.2	20.0 ± 0.1
Prasinoxanthin	nd	nd	nd	nd	nd	22.64 ± 0.20	41.13 ± 0.2
Violaxanthin	54.60 ± 2.3	83.01 ± 11.7	1228 ± 61	nd	1679 ± 83	nd	nd
19-hex-fuco	nd	nd	nd	nd	nd	nd	nd
Dinoxanthin	nd	nd	nd	nd	nd	nd	nd
Antheraxanthin	nd	344.4 ± 4.6	nd	nd	nd	nd	nd
Astaxanthin	nd	nd	nd	nd	nd	48.42 ± 0.5	nd
Diadinoxanthin	256.7 ± 3	43.44 ± 1.0	nd	2166 ± 68	140.5 ± 2.4	nd	nd
Alloxanthin	17.64 ± 0.1	55.73 ± 0.5	nd	nd	130.5 ± 2.9	94.06 ± 0.8	24.33 ± 0.1
Diatoxanthin	26.75 ± 1.0	nd	136.3 ± 1.0	1558 ± 88	nd	nd	nd
Lutein	5111 ± 61	3014± 76	nd	nd	nd	3220 ± 54	2069 ± 34
Zeaxanthin	284.5 ± 2.5	195.8 ± 6.7	136.8 ± 1.0	nd	584.9 ± 3.6	151.1 ± 1.7	15.52 ± 1.0
Dihydro lutein	145.0 ± 2.9	175.6 ± 2.5	nd	216.2 ± 2.0	nd	248.7 ± 9	111.1 ± 17
Unknown	nd	nd	nd	nd	165.2 ± 3.5	nd	nd
Chlorophyll b	862.6 ± 8.6	1454 ± 23	nd	nd	nd	725 ± 35	389.3 ± 19
Chlorophyll a	2993 ± 14	3424 ± 87	1065 ± 22	2714 ± 23	2001 ± 54	615.3 ± 4.1	1455 ± 3.9
β-carotene	647.3 ± 13	743.5 ± 44.3	284.5 ± 2	348.7 ± 4.4	2223 ± 88	1039 ± 17	614.4 ± 8.3
Canthaxanthin	nd	nd	3.40 ± 0.05	nd	136.5 ± 12.1	nd	nd
α-carotene	nd	76.01 ± 0.1	nd	nd	84.16 ± 3.2	nd	nd

Values are given as mean (n = 2) ± standard deviation (absolute value). nd = Not detected.

2.5. Antioxidative Properties

2.5.1. ABTS-Radical Scavenging Capacity (TEAC)

This method monitors the ability of antioxidant compounds to interfere with the reaction between peroxy radicals. This assay involves the initiation of peroxidation by generating water-soluble peroxyl radicals and is sensitive to all known chain breaking antioxidants such as phenolics and carotenoids [43].

Highest TEAC radical scavenging activity in this study was detected in De.S and the lowest in P.T. There was no statistically significant difference between concentrations 0.25 mg/mL and 0.5 mg/mL. (Table 4). Results of radical scavenging for C.S1, which was exposed to higher light

intensity was higher than for C.S2, which can be explained by the higher content of carotenoids in this sample. Based on the results of analysis of variance, phenolic compounds showed highest contribution to the ABTS radical scavenging capacity, followed by carotenoid contents. We estimated the correlation of independent variables including tocopherols, phenolic compounds, and carotenoids to the results of this assay. The following fitted model was calculated and can explain R^2 = 86.06% of the variability in assay's results.

$$\text{TEAC} = -1.28 + 1.13\,(\text{Phenolic Compounds}) + 0.343\,(\text{Carotenoids}) + 0.0017\,(\text{Tocopherols})$$

Results of the multivariate data analysis, which was done by the Partial Least Squares (PLS) method, also confirmed that phenolic compounds and carotenoids were major contributors to the ABTS radical scavenging capacity in microalgae methanol extracts (Figure 3). Carotenoids, such as lutein and zeaxanthin, are known for their ability to quench the radicals and singlet oxygen [44], so this can explain the contribution of carotenoids in this radical scavenging assay. This assay have been used previously by many different studies to evaluate the microalgae radical scavenging capacity [3,16] and our results are in agreement with the results of these reports.

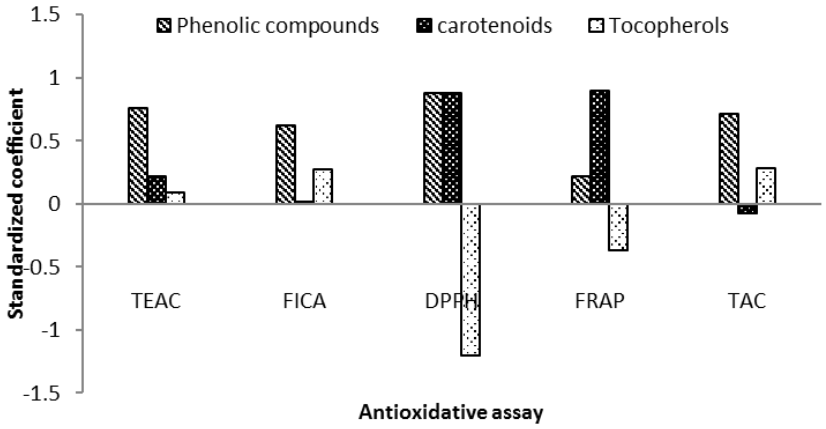

Figure 3. PLS coefficient plots. Bars represent the standardized correlation coefficients of predictor variables (phenolic compounds, tocopherols, and carotenoids) for each response variable (antioxidative assay).

2.5.2. Ferrous Ion-Chelating Ability (FICA)

The FICA is a measure of chelating ability of the ferrous ion, which is important to avoid reactions that could lead to development of radicals such as hydroxyl. The following multiple regression model was calculated to show the correlation of phenolics, carotenoids, and tocopherols to the results of ferrous ion chelating ability.

$$\text{FICA} = 3.26 + 1.42\,(\text{Phenolic Compounds}) + 0.046\,(\text{Carotenoids}) + 0.008\,(\text{Tocopherols})$$

The R-squared statistics indicates that the fitted model explains 68.56% of the variability in chelating ability. Analysis of variance confirmed that phenolic compounds is the main contributor to the results (F = 2.74, p = 0.02). Multivariate data analysis based on PLS coefficient method also confirmed the order of contribution as phenolic compounds and tocopherols, respectively (Figure 3). Highest chelating ability was detected in De.S (1 mg/mL) and lowest activity in P.T (0.25 mg/mL). Chelating ability in sample of *Chlorella sorokiniana*, which was exposed to higher light intensity (C.S1), was lower, compared to C.S2 (Table 4). Our result based on this assay confirmed some previous studies [9,13].

Table 4. Result of evaluation of antioxidative properties.TEAC (Trolox equivalent/g). FICA (% chelating). DPPH (% inhibition). FRAP (mg/g Ascorbic acid equivalent) and TAC(GAE/g).

Concentration * TEAC	**De.S a**	**Du.S c,d**	**N.L c,d**	**P.T e**	**N.S a,b**	**C.S1 d**	**C.S2 b,c**
1.0 a	24.26 ± 0.60	14.38 ± 0.40	16.60 ± 0.20	6.79 ± 0.02	20.34 ± 0.15	18.75 ± 0.16	13.48 ± 0.38
0.50 b	10.05 ± 0.30	6.58 ± 0.03	5.48 ± 0.23	3.35 ± 0.10	8.68 ± 0.10	6.32 ± 0.11	7.30 ± 0.20
0.25 b	7.05 ± 0.10	5.01 ± 0.05	5.16 ± 0.01	2.73 ± 0.20	6.66 ± 0.14	5.24 ± 0.14	6.80 ± 0.010
Concentration FICA	De. S a	Du.S c	N.L d	P.T e	N.S b	C.S1 c,d	C.S2 b
1.0 a	20.10 ± 0.51	13.99 ± 0.18	11.61 ± 0.97	9.67 ± 0.60	16.90 ± 0.14	12.15 ± 0.042	17.91 ± 0.21
0.5 b	16.87 ± 0.60	11.77 ± 0.31	9.45 ± 0.49	7.55 ± 0.21	14.25 ± 0.35	10.57 ± 0.60	14.92 ± 0.17
0.25 c	8.57 ± 0.17	5.42 ± 0.17	3.95 ± 0.21	3.35 ± 0.21	7.17 ± 0.10	5.40 ± 0.14	7.53 ± 0.19
Concentration DPPH	De. S c	Du.S d	N.L a,b	P.T d	N.S b	C.S1 a	C.S2 c,d
1.0 a	29.11 ± 0.01	26.95 ± 0.10	35.17 ± 0.08	28.35 ± 0.07	30.32 ± 0.02	34.09 ± 0.08	28.06 ± 0.04
0.5 b	18.24 ± 0.14	15.55 ± 0.21	19.44 ± 0.14	15.58 ± 0.01	21.26 ± 0.08	26.05 ± 0.07	15.38 ± 0.26
0.25 c	10.29 ± 0.24	12.66 ± 0.09	14.27 ± 0.09	8.41 ± 0.12	12.55 ± 0.07	11.74 ± 0.05	9.34 ± 0.15
Concentration FRAP	De.S a	Du.S b,c	N.L e	P.T d	N.S a	C.S1 c,d	C.S2 b
1.0 a	0.45 ± 0.01	0.31 ± 0.02	0.30 ± 0.01	0.27 ± 0.01	0.39 ± 0.021	0.32 ± 0.01	0.35 ± 0.01
0.5 b	0.32 ± 0.01	0.27 ± 0.01	0.16 ± 0.01	0.26 ± 0.01	0.35 ± 0.02	0.25 ± 0.01	0.27 ± 0.01
0.25 c	0.25 ± 0.01	0.25 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.30 ± 0.01	0.18 ± 0.01	0.22 ± 0.01
Time ** TAC	De.S a	Du.S c	N.L b	P.T d	N.S a	C.S1 c	C.S2 c
60 a	5.22 ± 0.14	3.24 ± 0.13	2.65 ± 0.13	1.83 ± 0.01	6.30 ± 0.012	2.96 ± 0.08	3.47 ± 0.04
90 b	8.56 ± 0.21	5.17 ± 0.05	6.57 ± 0.41	2.55 ± 0.21	7.29 ± 0.13	4.32 ± 0.09	4.65 ± 0.16
120 b	**8.95 ± 0.01**	**5.27 ± 0.01**	**7.00 ± 0.02**	**2.99 ± 0.06**	**7.30 ± 0.14**	**4.31 ± 0.26**	**5.36 ± 0.06**

Values are given as mean (n = 3) ± standard deviation (absolute value). For each test the same letters indicate homogeneous values ($p < 0.05$). * Values in the first column show the concentration of methanolic extract for TEAC, FICA, DPPH, and FRAP (mg algae biomass dry weight/mL). ** Values in the first column show the reaction time (min) for TAC.

2.5.3. DPPH Radical Scavenging Activity Assay (DPPH)

This assay is based on the measurement of the reducing ability of antioxidants toward DPPH. The ability can be evaluated by electron spin resonance or by measuring the decrease of its absorbance. The 1,1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging activities of methanolic extracts increased with increasing concentration. Result of analysis of variance showed a statistically significant effect of both concentration and species on DPPH radical scavenging activity at the 95.0% confidence levels (Table 4). The highest activity was detected for N.L and C.S1 at 1 mg/mL and the lowest activity for P.T at concentration of 0.25 mg/mL (Table 4). There was a significant difference between the C.S1 and C.S2 samples, showing that the light intensity had a considerable effect on the radical scavenging property. A multiple linear regression model was fitted (R-squared = 69.86%) to describe the relationship between DPPH and independent variables including tocopherols, carotenoids, and phenolics as below.

$$\text{DPPH} = -1.62 + 2.39\,(\text{Phenolic Compounds}) + 2.36\,(\text{Carotenoids}) - 0.04\,(\text{Tocopherols})$$

Based on results of analysis of variance for the fitted model, both carotenoids and phenolic compounds showed strong correlation to the DPPH radical scavenging property of microalgae methanolic extracts. This finding was confirmed by using PLS correlation method, as shown in Figure 3. Contribution of the carotenoids to the radical scavenging activity was described by several researches previously [12,45,46]. Our results confirm that both carotenoids and phenolics are contributing to the radical scavenging property of microalgal methanolic extract, while the contribution of tocopherols was not estimated as significant.

2.5.4. Ferrous Reduction Power (FRAP)

The highest reducing power was detected in De.S (1 mg/mL) methanolic extract and the lowest in N.L (0.25 mg/mL). Both effects of concentration and species were evaluated as significant at 95% confidence level. Multiple comparison procedure based on Fisher's least significant difference (LSD)

procedure revealed that there was a statistical significant difference between concentrations, and strains (Table 4). The following regression model was calculated to describes the relationship between results of FRAP assay and three independent variables including carotenoids, phenolic compounds, and tocopherols.

$$\text{FRAP} = 0.059 + 0.0095\,(\text{Phenolic Compounds}) + 0.038\,(\text{Carotenoids}) - 0.00019\,(\text{Tocopherols})$$

There was a statistically significant relationship between the variables at the 95.0% confidence level. The *R*-Squared statistic explains 55.64% of the variability in the results of FRAP assay. Highest effect corresponded to carotenoids (T = 2.9 and p-value = 0.01) followed by phenolic compounds which can suggest the carotenoid as the main contributor to the ferrous reducing power. It was also confirmed by PLS test as shown in Figure 3. The effect of light intensity on the two *Chlorella* species was also evaluated as significant. The correlation between content of carotenoids to the ferrous reduction power was already reported before [13]. The antioxidant mechanism of carotenoids is mostly known as radical scavenging, so it can show the individual ability of carotenoids, such as lutein, to involve in single electron transfer-based reactions which are the basic principle in the analysis of ferric reducing power assay [44].

2.5.5. Total Antioxidant Capacity Assay (TAC)

This method can determine the antioxidant capacity, through the formation of phosphomolybdenum complex, and the reduction of Mo (VI) to Mo (V) by the antioxidant components in sample which would result in formation of a green phosphate Mo (V) complex at acidic pH. Reaction time is normally between 60 to 150 min, depending of the composition in the extracts as the formation of the complex is temperature-dependent for various reducing compounds, such as tocopherols and phenolic compounds. The analysis of variance with two factors (species and time of reaction) was used to evaluate the variability of total antioxidative capacity. Result showed a statistically significant effect of both variables on total antioxidative capacity at the 95.0% confidence levels. A multiple comparison procedure based on Fisher's LSD procedure revealed that there is a statistically significant difference between strains, while the difference for the reaction time was not significant between 90 and 120 minuutes (Table 4). Thus, the reaction can be completed in 90 min. Highest activity was detected for De.S at 120 min and lowest activity for T at 60 min with values of 8.95 $\pm$ 0.07 mg GAE/g and 1.83 $\pm$ 0.01 mg GAE/g, respectively. There was no significant difference between the C.S1 and C.S2 samples (Table 4).

A multiple linear regression model was calculated which describes the relationship between total antioxidative capacity and the three independent variables including carotenoids, phenolic compounds, and tocopherols. The equation of the fitted model was:

$$\begin{aligned}\text{Total Antioxidative Capacity} = {} & -0.011 + 1.0\,(\text{Phenolic Compounds}) - 0.10\,(\text{Carotenoids}) \\ & + 0.005\,(\text{Tocopherols})\end{aligned}$$

The *R*-Squared statistic indicates that the model as fitted explains 77.49% of the variability in total antioxidant capacity. Further analysis of variances showed that phenolic compounds have the main effect in results of total antioxidative capacity. It could be attributed to the fact that both methods (TAC and Folin) are based on the reaction of reducing compounds in the extract. We used gallic acid as a reference standard for both tests and this can be another reason for this finding. Contribution of tocopherols and carotenoids in the result of this assay were estimated as weak and not significant. Further confirmation of the results was done by estimation of correlations by PLS coefficient at 95% confidence level. As shown in Figure 3, phenolic compounds were the main correlating factor for total antioxidative capacity assay in microalgae samples. The same result was reported previously [34] while another study reported the contribution of both carotenoids and phenolic compounds [7].

2.6. Contribution of Carotenoids in Antioxidative Activity of Microalgae Biomass

Our results showed that carotenoids contribute significantly to some antioxidant properties of microalgae species grown on industrial waste water. The contribution of carotenoids in antioxidant activity of microalgae extracts was evaluated in some previous studies [9,34,47].

The antioxidative power of carotenoids is not the same. The electron-rich conjugated system of the polyene and functional cyclic end groups determine the antioxidant activities of carotenoids together [47]. Ketocarotenoids, including astaxanthin and canthaxanthin, can be found mostly in algae. Epoxy carotenoids such as antheraxanthin, violaxanthin and fucoxanthin are also abundant in algae. It has been shown that pigments like astaxanthin, β-carotene, lutein, neoxanthin, and also zeaxanthin have a scavenging property [47], while astaxanthin has been claimed to show the highest effect among all carotenoids. Scavenging function of carotenoids against peroxyl radicals (ROO*) was reported as even stronger than α-tocopherol with order of astaxanthin > lutein > zeaxanthin > α-tochopherol > fucoxanthin > β-carotene while in relation to the hydroxyl radical (HO) scavenging capacity the order of strength was reported as β-carotene > lutein > zeaxanthin > astaxanthin > α-tochopherol [47]. It was claimed that the *in vitro* antioxidant property of astaxanthin was 10 times stronger than zeaxanthin, lutein, tunaxanthin, canthaxanthin, and β-carotene, and even 100 times stronger than α-tocopherol [48]. In our study both composition and content of carotenoids varied significantly between the species. Main carotenoid compound(s) in green algae including De.S, Du.S, C.S1, and C.S2 was lutein, while in P.T, and *Nannochloropsis* samples the main carotenoids were diatoxanthin and diadinoxanthin, and violaxanthin and β-carotene, respectively.

2.7. Effects of Source of Nitrogen on Productivity of Biomass and Bioactive Compounds

Nitrogen is the most important nutrient for the growth of the algal biomass, and is a key constituent of many algal cellular components. Most microalgae species are capable of utilizing a variety of inorganic nitrogen (e.g., ammonia, nitrate, and nitrite, *etc.*) [46], so waste water from different sources such as industrial and municipal activities could be used as a good nitrogen sources. In the industrial wastewater we used in this study the majority of nitrogen is in the form of ammonia. Among the above nitrogen forms, ammonium is the most preferred form of nitrogen source for some microalgae in part because its uptake and utilization by microalgae is most energy efficient [11,21]. It is commonly accepted that nitrogen metabolism is linked to carbon metabolism in algae because they share organic carbon and energy supplied directly from photosynthetic electron transport and CO2 fixation as well as from the metabolic pathway of organic carbon [23,46]. Biomass is the main product of a microalgae cultivation system [24], and productivity of intracellular bioactive compounds, such as carotenoids and phenolics depends directly on the productivity of the biomass. The literature shows that the cell growth rate, lutein content, and lutein productivity of some microalgae are mainly influenced by the culture conditions, such as light intensity and nitrogen concentration [6,34,42]. Green algae have a fast growth rate with a good compatibility to the growth conditions and for this reason they have a wide potential for large scale cultivation, because of their robustness and simple nutritional requirements, so the higher concentration of carotenoids (lutein) in fresh water *Desmodesmus* sp. could be correlated to this feature. In addition to light, which is the main energy source of microalgae, chemicals including carbon dioxide, inorganic nitrogen (ammonia or nitrate), and phosphate are required for photoautotrophic growth. Production of lutein in *Chlorella sorokiniana* or *Desmodesmus* sp. at higher concentrations has been demonstrated before [41,42]. For the higher production of lutein, in addition to the overexpression of specific enzymes, additional storage space (outside of the photosystem) needs to be created. Therefore, *Desmodesmus* sp. and *Chlorella sorokiniana* are suitable strains for lutein production by using industrial wastewater as main nutrient source.

3. Experimental Section

3.1. Chemicals and Reagents

Standards of phenolic compounds and tocopherols were purchased from Sigma (St. Louise, MO, USA) and Fluka (Deisenhofen, Germany) and standards of pigments were purchased from DHI (Hørsholm, Denmark). HPLC grade acetonitrile, heptane, isopropanol, methanol, and acetone were purchased from Sigma and Fluka. HPLC grade water was prepared at DTU Food using Milli-Q® Advantage A10 water deionizing system from Millipore Corporation (Billerica, MA, USA).

3.2. Microalgae Biomass

Microalgae strains *Nannochloropsis salina.* (*Strain Number: 40.85*) and *Nannochloropsis limnetica* (*Strain Number: 18.99*) were obtained from the culture collection of algae (SAG), University of Gottingen. Strain of *Desmodesmus* sp. (De.S) was isolated from waste water treatment system, Kalundborg Kommune and identified by Dr Gert Hansen, Department of Biology, University of Copenhagen. *Chlorella sorokiniana* (C.S1 and C.S2) were cultivated in flat panel reactors at two light intensities, 2000 µmol photon $m^{-2} \cdot s^{-1}$ and 200 µmol photon $m^{-2} \cdot s^{-1}$, respectively, as in [49], *Phaeodactylum tricornutom* (P.T) and *Dunaliella salina* (Du.S) were cultivated at DTU Environment in 10 L Schott bottles, stirred with magnets and aerated with 2% carbon dioxide/air mixture under fluorescent lights with intensity 300 µ mol photon $m^{-2} \cdot s^{-1}$. *Nannochloropsis salina* (N.S), *Desmodesmus* sp. and *Nanochloropsis limnetica* (N.L) were cultivated at DTU Food in 5 L Schott bottles, stirred with magnets and aerated with 2% carbon dioxide/air mixture under fluorescent lights with intensity 200 µmol photon $m^{-2} \cdot s^{-1}$. Industrial waste water was obtained from Kalundborg municipality and used as main nutrient source for all cultivations. Table 5 shows specification of industrial wastewater. Microalgae biomass was harvested at stationary phase by a pilot scale (100 L/h) ceramic membrane microfiltration unit with one micron pore size membrane (Liqtech International, Ballerup, Denmark) and then immediately freeze dried. Dried samples were stored at −20 °C until analysis.

Table 5. Chemical composition of industrial wastewater which was used as main nutrient source.

Item	Unit	Amount
pH	-	8.1
Suspended solids	mg/L	20
Total N	mg/L	190
Ammonia + ammonium-N	mg/L	150
Nitrite + nitrate	mg/L	<0.1
Total P	mg/L	11
Sulphate	mg/L	3.6
Total cyanide	µg/L	2.5
Total Alkalinity	mmol/L	62.5
EDTA	mg/L	<0.5
Sodium(Na)	mg/L	1500
Cadmium (Cd)	µg/L	<0.05
Copper (Cu)	µg/L	3.4
Iron (Fe)	mg/l	0.23
Cobolt (Co)	µg/L	<0.5

3.3. Sample Preparations

3.3.1. Antioxidative Properties, Tocopherols, and Phenolic Compounds (HPLC)

Freeze-dried samples were ground into a fine powder, and then 50 mg samples were soaked in 5 mL of pure methanol and shaken vigorously for 30 s. Then tubes were put in sonication bath (Branson Corp., Danbury, CT, USA) in the dark and at room temperature for 45 min. Then, all samples

were centrifuged at 7500 *g* for 10 min and supernatants were separated. The extraction process was repeated with another 5 mL portion of pure methanol. Collected supernatants were combined and stored at −20 °C. For analysis of tocopherols, one milliliter of the extract was evaporated under a stream of nitrogen and then re-dissolved in one milliliter of *n*-heptane. For analysis of phenolics and antioxidative properties tests, the rest of the methanolic extract solution was diluted with pure methanol to various concentrations (mg algae biomass dry weight/mL) for each test.

3.3.2. Pigments

Freeze-dried samples were ground into a fine powder, after which 20 mg samples were soaked in 5 mL of methanol containing 0.025 μg/mL BHT as internal standard and antioxidant. Then, the tubes were shaken vigorously for 30 seconds and put in sonication bath at a temperature lower than 5 °C for 15 min. Subsequently, the tubes were centrifuged at 5000 *g* and the supernatants were separated. The extraction was repeated with 5 mL portion(s) of solvent until a nearly colorless biomass was obtained. Supernatants were combined and used immediately for the analysis.

3.4. Analytical Methods

3.4.1. Total Phenolic Content

The total phenolic content of the algae extracts was determined via a modified Folin–Ciocalteu method as described by Choochote *et al.* [9]. Briefly, 100 μL of diluted extract solution (1 mg/mL) was mixed with 0.6 mL of deionized water and 0.5 mL of Folin-Ciocalteu reagent in a test tube and then 1.5 mL of 20% sodium carbonate aqueous solution was added and the volume was made up to 10 mL with deionized water. The samples were incubated for 30 min at room temperature in darkness and then absorbance of the reaction mixtures were measured at 760 nm. Gallic acid was used as a standard and the total phenolic content of the extracts were expressed in milligram gallic acid equivalent.

3.4.2. Phenolic Compounds (HPLC)

Extracts (2.3.1) were filtered prior to the analysis by 0.22 μm PVDF syringe filter, and then analyzed by HPLC using an Agilent 1100 Liquid Chromatograph (Agilent technologies, Santa Clara, CA, United States) equipped with a DAD. The separation was carried out on a Prodigy ODS-3 column 250 mm, 46 mm with 5 μm particle size from Phenomenex (Torrance, CA, USA). Injection volume was 20 μL and the mobile phase was a mixture of solvent A (phosphoric acid in de-ionized water, pH = 3) and solvent B (acetonitrile) at 0.9 mL/min. The gradient started with 5% of B and after 2 min increased to 40% in 20 min and again increased to 100% B at 15 min and finally kept constant for 25 min. Total acquisition time was 70 min. Detection was done at 280 nm. The identification of the peaks was done using standards which include gallic acid, 2,5-dihydroxy benzoic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, catechin hydrate, ginnestein, 4-hydroxy benzoic acid, caffeic acid, syrringic acid, *p*-coumaric acid, ferulic acid, *O*-salicylic acid, and cinnamic acid. Total of identified phenolics was also calculated.

3.4.3. Total Carotenoids and Pigment Composition

The extracts (2.3.2) were filtered prior to the analysis by methanol compatible 0.22μm PTFE syringe filter and then analyzed by HPLC using an Agilent 1100 Liquid Chromatograph equipped with a DAD. The separation was carried out in a Zorbax Eclipse C8 column 150 mm, 46 mm with 3.5 μm particle size from Agilent. The chromatographic separation was carried out according to the method described by Van Heukelem *et al.* [50] with modifications. The temperature of injection port was 5 °C.

The mobile phase was a mixture of solvent A (70% methanol + 30% of 0.028 M tertiary butyl ammonium acetate in water) and solvent B (methanol) at a flow rate of 1.1 mL/min. The gradient program was started with 5% of B and then increased to 95% in 27 min, kept constant for 7 min and

then changed to 100% in one minute and kept constant for 5 min. Total acquisition time was 40 min. The temperature in the injection port was kept constant at 5 °C and the sample was mixed with the buffer (0.028 M tertiary butyl ammonium acetate in water) at the proportion of 1:3 for 3 min just prior to the injection. Identification of peaks and calibration was done by individual standards for each pigment. Detection was done at 440 nm for pigments and 280 nm for BHT as internal standard. Sum of the carotenoids was calculated as total carotenoids.

3.4.4. Total Flavonoids

Total flavonoids content in algae extracts was determined by the method described by Sava *et al.* [29], with some modifications. To 20 µL of algal extract, 20 µL 10% $AlCl_3$ and 20µL 1 M potassium acetate plus 180 µL of distilled water was added, and then tubes were kept at room temperature for 30 min. Optical density was measured at 415 nm against blank. The calibration curve was made by quercetin prepared in methanol. Results expressed as milligrams of quercetin equivalent per gram of sample.

3.4.5. Total Tocopherols

One milliliter of the methanolic extract was evaporated to dryness in darkness and under a stream of nitrogen and then re-dissolved in a mixture of isopropanol: heptane (0.5:99.5, v/v). Then the solution was filtered by suitable 0.22 µm PTFE syringe filter and 20 µL of filtrate was injected to HPLC. Analysis was done based on the AOCS official method as [51] using an Agilent 1100 Liquid Chromatograph equipped with a fluorescence detector, with the excitation wavelength set at 290 nm and emission wavelength at 330. The separation was carried out in isocratic mode by Spherisorb column 150 mm, 46 mm with 3 µm particle size, using a mixture of isopropanol: n- heptane (0.5:99.5, v/v) as mobile phase.

3.4.6. Total Antioxidant Capacity Assay (TAC)

The total antioxidant capacity assay of the microalgae extracts was determined by the method of Pan *et al.* [52] with some modifications. 300 µL of diluted extract solution (0.5 mg/mL) were added to a test tube containing 3 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate plus 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 60, 90 or 120 min. Then the mixtures were cooled to room temperature and absorbance was measured at 695 nm against water as blank. Gallic acid was used as the reference standard.

3.4.7. DPPH Radical Scavenging Activity Assay (DPPH)

The method used for measuring the DPPH radical scavenging ability of the algae extracts was that of Choochote *et al.* [9]. Various concentrations (0.25, 0.5, and 1 mg/mL) of the extract was made with pure methanol and then 100 µL of each extract was added to 2 mL of DPPH (0.5 mM in absolute ethanol), respectively. The mixtures were shaken vigorously and then left to stand at room temperature for 30 min in the dark. The absorbance was measured at 517 nm against a methanol extract/water extract blank. BHT was used as the reference standard and results compared as the percent of inhibition against 100% of inhibition. The percent inhibition (I %) was calculated using the formula:

$$\mathrm{I}\,\% = ((\mathrm{Abs\,control} - \mathrm{Abs\,sample})/\mathrm{Abs\,control}) \times 100$$

3.4.8. Ferrous Ion-Chelating Ability (FICA)

The ferrous ion-chelating ability was determined according to the method of Duan *et al.* [31]. The extract solution was diluted with pure methanol to various concentrations (0.25, 0.5, and 1 mg/mL) and then 2000 µL of each was mixed with 2.7 mL distilled water, FeCl2 (0.1 mL, 2 mM) and ferrozine (0.2 mL, 5 mM). Then, the solutions were incubated for 10 min in dark and at room temperature. After incubation, the absorbance was measured at 562 nm. Deionized water (2 mL) was

used instead of sample as a control, and instead of ferrozine solution as a blank. EDTA (1 mg/mL) was used as reference. The ferrous ion-chelating ability was calculated as follows:

Chelating ability (%) = $[A_0 - (A_1 - A_2)/A_0] \times 100$, where A_0 is the absorbance of the control, A_1 the absorbance of the sample or EDTA, and A_2 is the absorbance of the blank.

3.4.9. Ferrous Ion Reduction Power (FRAP)

Reducing power of algae extracts were determined by the method of Benzie *et al.*, [53]. In brief, 1.0 mL of diluted extracts (0.25, 0.5 and 1 mg/mL) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferric cyanide (1%). Reaction mixture was kept in a water bath at 50 °C for 20 min. After incubation, 2.5 mL of trichloroacetic acid (10% of TCA) was added and tubes were centrifuged at 2000 rpm for 10 min. From the upper layer, 2.5 mL solution was mixed with 2.5 mL distilled water and 0.5 mL of FeCl3 (0.1%). Absorbance of all the solution was measured at 700 nm. Ferric reducing antioxidant power is expressed as the reducing power compared to ascorbic acid (1 mg/g) as reference standard.

3.4.10. ABTS-Radical Scavenging (TEAC)

The ABTS radical scavenging activity was determined according to the method described by Li *et al.* [3]. For the assay, $ABTS^+$ radical cation was generated by preparing a solution of 7 mM ABTS and 2.45 mM potassium persulphate in deionised water. The reaction mixture was allowed to stand in the dark for 16 h at room temperature and was used in the same day. The $ABTS^+$ solution was diluted with deionised water to give an absorbance of 0.700 $\pm$ 0.050 at 734 nm. The extract solution was diluted with pure methanol to various concentrations (0.25, 0.5 and 1 mg/mL) and then 100 µL of each diluted sample were mixed with 1.9 mL of diluted $ABTS^+$ solution. After 10 min dark incubation at room temperature, the absorbance was measured at 734 nm. Trolox (0–25 µM) was used as a reference standard. Controls were included de-ionized water and ammonium acetate buffer instead of the reagents and pure de-ionized water and 96% ethanol instead of sample.

3.4.11. Statistical Analyses

Measurements were carried out in triplicate unless otherwise stated and the results are given as mean values $\pm$ absolute standard deviations. Results were compared using ANOVA test with least squares' post-test with significance level $\alpha = 0.05$. Multiple regression and multivariate data analysis (partial least squares coefficient method) were also carried out to evaluate and demonstrate the effects of carotenoids, phenolic, and tocopherols as predictor variables on each individual antioxidative property test as response variable. Partial least squares coefficient graphs were used to show significance and the magnitude of the relationship between predictors and responses. All statistical analyses were done by STATGRAPHICS-centurion XVI software from Statpoint Technologies (Warrenton, MO, USA).

4. Conclusions

This study evaluated the antioxidative properties of microalgal methanolic extract by means of different assays and correlated the results to the content of some natural antioxidants which were present in the microalgae biomass. Phenolic compounds contributed to all the antioxidative properties measured while the contribution of carotenoids to these properties was confirmed for the 1,1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging activity assay, ferrous reduction power (FRAP) and ABTS-radical scavenging capacity. Tocopherols did not appear to contribute to the antioxidative activities to a significant extent.

The study focused on main natural antioxidants in the microalgae biomass while there are many other compounds which are known as antioxidants such as amino acids, polysaccharides, and quinoid compounds which could affect the antioxidative properties in microalgae biomass.

Desmodesmus sp. which was isolated from waste water treatment facility in Kalundborg, Denmark, produced the highest amounts of pigments, phenolics, and tocopherols and had the best antioxidative properties. *Phaeodactylum tricornutom* species showed the poorest antioxidative properties and had the lowest amounts of antioxidants and the highest accumulation of beta-carotene was observed in *Nannochloropsis salina*.

The effects of light intensity for one species (*Chlorella sorokiniana*) was also evaluated, showing that high light intensity could improve the development of carotenoids while it could have adverse or no effects on the antioxidative properties. The industrial processing waste water which was used in this study contained very low concentrations of heavy metals and hazardous materials (Table 5), and could represent a suitable and feasible source of nutrient for the production of bioactive compounds such as pigments and tocopherols.

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Paper 2

Two-step direct transesterification as a rapid method for the analysis of fatty acids in microalgae biomass

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Abstract:	A rapid and routine direct transesterification (DT) method for the evaluation of the fatty acid composition of microalgae biomass is presented in this paper. The method was optimised on Lake Superior Fish Tissue (SRM® 1946), as standard reference material and then the proposed DT procedure was tested on various microalgae species, and the results were compared to a validated microwave assisted esterification (MAE) as a reference (R- squared = 0.99). Total DT time was very fast (14 minutes), while the fatty acid compositions were virtually identical to those prepared by MAE for various species of microalgae as well as fish and krill biomasses.

Research Article

Two-step direct transesterification as a rapid method for the analysis of fatty acids in microalgae biomass

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Abbreviations:

FAME; fatty acid methyl esters, DT; direct transesterification, MAE; microwave assisted esterification, BF3; boron tri fluoride.

Abstract

A rapid and routine direct transesterification (DT) method for the evaluation of the fatty acid composition of microalgae biomass is presented in this paper. The method was optimised on Lake Superior Fish Tissue (SRM® 1946), as standard reference material and then the proposed DT procedure was tested on various microalgae species, and the results were compared to a validated microwave assisted esterification (MAE) as a reference (R-squared = 0.99). Total DT time was very fast (14 minutes), while the fatty acid compositions were virtually identical to those prepared by MAE for various species of microalgae as well as fish and krill biomasses.

Practical applications

Microalgae are known as a sustainable source of bioactive compounds such as fatty acids, and their implementation is growing. Application of microalgae biomass as a source of fatty acids requires fast and reliable methods of analysis of fatty acids. The direct transesterification method developed in this study could be used as a powerful tool for the monitoring of the variations in fatty acid composition during the cultivation, processing, formulation and storage.

Introduction

Microalgae are known as a sustainable source of lipids including long-chain polyunsaturated fatty acids (LC-PUFA), which are critically required for applications such as aquatic feed production (Safafar et al., 2016). Application of microalgal lipids in food, feed or cosmetics requires fast, reliable and precise methods for quality control and for monitoring the deterioration during storage, or processing (Willers et al. 2015). Various methods have been developed for the analysis of fatty acid composition in microalgae biomass. The methods can be divided into two core groups as indirect and direct methods (Qiao et al. 2015). Indirect methods require solvent extraction of lipids and subsequent transesterification of fatty acids in the extracted lipid fraction. Lipid extraction is mostly being done with a combination of chloroform and methanol(Bligh&Dyer 1959; Folch et al. 1957) or hexane. The organic solvent(s) are then evaporated to dryness (e.g. under a gentle stream of nitrogen) and the lipid extract is esterified and analysed by gas chromatography. In the Folch method a 8:4:3 ratio of chloroform, methanol and water are being used, while Bligh & Dyer solvent includes a ratio of 10:10:9 (or, traditionally, 2:2:1.8) for chloroform: methanol: water. It has been shown that the solvent extraction could not extract all of the lipids in the biomass (Palmquist& Jenkins 2003). On the other hand, during the solvent extraction, other non-lipid compounds such as pigments (chlorophylls, phaeopigments and carotenoids) could also be extracted and may interfere in the subsequent esterification reaction (Qiao et al. 2015). For samples which include more than 2% DW of lipids, Bligh & Dyer method may underestimate the lipid contents, and this underestimation increased significantly with increasing lipid contents (Iverson et al., 2001). Through direct methods, *in situ* derivatization of the fatty acids to fatty acid esters, is carried out in the biomass, followed by the extraction of fatty acids esters, which are then analysed by gas chromatography. DT methods require less sample, solvent and reaction time, while their efficiency is higher than direct methods (Carrapiso and García 2000; Johnson andWen 2009; Griffiths et al. 2010; Soares et al. 2014; Chu et al. 2015; Qiao et al. 2015). It was demonstrated that DT of fatty acids in microalgae biomass yielded higher fatty acid esters than

conventional methods, due to less washing/evaporation and higher reaction efficiency (Johnson and Wen 2009; Griffiths et al. 2010). The two main concerns in performing *in situ* reactions are lipid solubilization and prevention of water or other compounds which may interfere with the catalytic reactions (Carrapiso and García 2000). Acidic, basic or both catalysts may be used for one or two-step derivatization. In base-catalyzed transesterification, the interference of water is higher than acid-catalyzed transesterification, because free fatty acids resulting from lipid hydrolysis are not methylated properly (L'epage and Roy 1986), so only a few methods have used base-catalyzed reagents for DT (Park and Goins 1994). L'epage and Roy (1986) developed a DT method for various biological matrices. The sample was heated at 100°C for 60 min in a mixture of methanol-benzene (4:1, v/v), in the presence of acetyl chloride. This method, after modification, was used for selective methylation of free fatty acids in plasma. In a DT method developed by Park & Goins (1994), a two-step esterification process using NaOH (0.5 N in methanol) and BF3 (14 % w/V in methanol) was used. Samples were heated at 90℃ for 10 minutes at each step. The efficiency of the method was comparable to standard indirect procedures. It was shown that the combination of acidic and basic catalysts could be more efficient than when each of them was used individually, particularly in the presence of water (Griffiths et al. 2010). Qiao et al. 2015, introduced a two-step DT method using basic (NaOH) and an acidic (acetyl chloride) catalysts. The results demonstrated good recovery yield of fatty acids in *Phaeodactylum tricornutum* compared with the traditional indirect methods. Dong et al. (2015), also developed a two-step DT method using alkaline (14 M KOH in methanol) and acidic (12 M H2SO4 in methanol) catalysts. After addition of catalysts, sample solutions were heated for 15 min at 85 °C. Authors indicated no significant differences in FAME quantification between the standard method (AOAC 991.39) and the proposed wet DT method.

Short time microwave irradiation could improve the efficiency of lipid extraction by solvent (Paré et al. 1997; Batista et al., 2001). This method has also been successfully used for the preparation of FAME(Patil et al. 2011; Cancela et al. 2012; Brunton et al. 2015). It has been proven that this technique speeds up the reactions, while the fatty acid profiles for MW assisted and conventionally prepared FAME were similar for various food products(Brunton et al. 2015). This study aimed at developing a DT procedure for rapid and routine assessment of fatty acid profile in microalgae. The method should combine the advantages of using a two step DT with those of using microwave assisted extraction of the fatty acids. It has been shown that the alkali-catalyzed methanolysis step for the determination of long-chain fatty acids produces dimethyl and mixed-alcohol esters from the plasticizer dioctyl [actually di(2-ethylhexyl)] phthalate. An independent boron trifluoride-catalyzed methanolysis step has proven to produce a lower level of artifacts, but

a two-step process produces a higher conversion than either catalyst did independently(Shantha and Ackman, 1991). To the best of our knowledge, this approach has not been attempted before. The results of DT on fatty acid composition of various algal species and some other marine samples were compared to those obtained by extraction using the Bligh and Dyer method followed by the standard transesterification procedure.

Materials and methods

Microalgae biomass

Microalgae wet biomass from *Nannochloropsis salina*, *Desmodesmusn* sp., *Chlorella pyrenoidosa*, *Chlorella sorokiniana* cultured on wastewater was prepared as described in Safafar et al. (2016). Spray dried *Spirulina* sp. and *Chlorella sorokiniana* were obtained from Ecoduna Productions-GmbH (Bruck an der Leitha, Austria). Spray dried *Nannochloropsis* sp., *Isochrysis* sp., and *Tetraselmis* sp. were provided by Necton company. (Olhão, Portugal). Krill meal was obtained from Olympic Seafood and salmon's flesh was obtained from the local supermarket.

Chemicals and reference materials

Standards of fatty acids were purchased from Sigma (St. Louise, USA) and Fluka (Deisenhofen, Germany). Lake Superior Fish Tissue (SRM® 1946) was purchased from National Institute of Standard and Technology, NIST (Gaithersburg, Maryland, USA). Heptane, methanol, toluene and BHT (butylated hydroxytoluene) and boron trifluoride(BF_3) were purchased from Sigma. HPLC grade water was prepared at DTU Food using Milli-Q® Advantage A10 water deionizing system from Millipore Corporation (Billerica, MA, USA).

Reference indirect transesterification

Lipids were extracted with chloroform, methanol, and water, as described by Bligh and Dyer (1959), using 200 mg of sample and a reduced amount of solvent, but keeping the original ratio between chloroform, methanol and water. The B&D extract was used for subsequent transesterification using the microwave assisted esterification method.

Microwave assisted esterification (MAE)

Around 1.0 g of Bligh and Dyer extract was weighed in a glass tube and then evaporated to dryness under a gentle flow of nitrogen. 100 µL of internal standard (IS) solution (2% w/v C23:0 FAME in heptane) was added and the reagents were mixed for two minutes on a whirly-mixer. Then 200 µL of heptane with BHT (0.01% w/v), 100 µL of toluene and one mL of boron trifluoride in methanol (14% w/v BF_3- MeOH) was added. Samples were mixed and methylated in the microwave oven (Microwave 3000 SOLV,

Anton Paar, city, country) for 10 min at 100±1°C and power of 500 W. Tubes were then immediately cooled down to room temperature and one mL of saturated salt solution (NaCl in deionized water) and 0.70 mL of heptane (0.01% w/v BHT) were added. The upper phase (0.7±0.05 mL) was transferred into GC vials for GLC separation. This method was previously developed and validated at DTUFood for the analysis of fatty acids in LC-PUFA containing marine samples.

Experimental design for direct transesterification (DT) procedure

Direct esterification procedures were optimised and compared to a standard reference material (SRM® 1946). The experimental design for the selection of the DT method is shown in **Figure 1**. The SRM® 1946 was used for the method selection and then the method was tested on various microalgae samples, and two samples of krill meal and salmon.

Chromatographic analysis (GLC-FID)

Chromatographic separation, identification and quantification were done according to the AOCS official method (Ce 1i-07) with some modification to shorten the analysis time, by gas chromatography system (HP-5890 A, Agilent Technologies, CA, USA). Fatty acid methyl esters were separated by the GC column DB 127-7012 (10 μm x 100 μm x 0.1 μm), from Agilent Technologies (CA, USA). Injection volume was 0.2 μL in split mode (1:70). The initial temperature of the oven was 160 °C. The temperature program set as follows: 160–200 °C (10.6 °C/ min), 200 °C kept for 0.3 min, 200–220 °C (10.6 °C/min), 220 °C kept for 1 min, 220–240 °C (10.6 °C/min) and kept at 240 °C for 3.8 min. The same procedure was used for both indirect reference transesterification, DT and MAE.

Statistics

All experiments and analyses were performed in two replications. Results are presented as mean ± std. Statistical procedures were carried out in STATGRAPHICS software, version Centurion XVII (Stat point Technologies Inc., Warrenton, VA, USA).

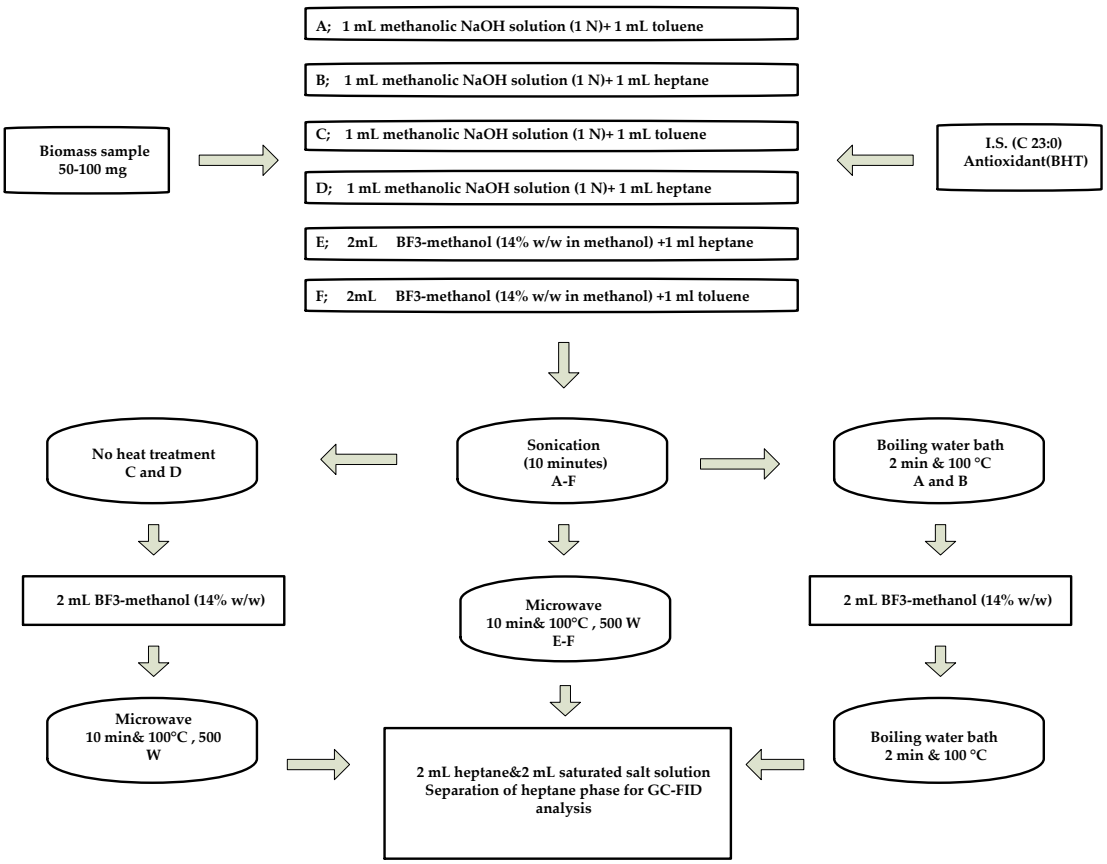

Figure 1. The experimental design for *in situ* transesterification of the fish tissue biomass. For all of the procedures, the SRM® 1946 was used. Procedures E and F treated by the single step microwave assisted esterification using just BF3(14% in methanol) as catalyst. In Procedures A, C and F, toluene was used in the initial mix, while for other (B, D and E), heptane was added instead. All samples were sonicated for 10 minutes, after addition of internal standard and antioxidant. Samples A and b were heated for 2 minutes at 100℃ , and in a boiling water bath, before and after addition of BF3 (14% in methanol), while samples C and D esterified in microwave after addition of methanolic BF3.

Results and discussion

Optimization and comparison of DT procedures

The proper sonication time was found using microalgae biomass from *Chlorella sorokiniana*, and results were compared to MAE as a reference (**Figure 2**). For no-microwave procedures, the optimised BF3 esterification time (2 minutes) and temperature (100℃) was obtained by using SRM® 1946 (**Figure 3**). For all major fatty acids including *C16:0*, *C16:1 n-7*, *C18:1 n-9*, *C20:5 n-3* and *C22:6 n-3* the highest concentration

were obtained by Procedure S1(100°C& 2 min), followed by S2(100°C& 4 min), S3(80°C& 2 min) and S4(80°C& 2 min). The microwave treatment conditions (10 minutes, 500 Watts) were optimised previously at DTU Food for the transesterification of marine samples. We observed no significant difference between the samples treated at various sonication times and of MAE, while significantly lower contents of FAME was achieved in the sample which was not treated by sonication. In the ultrasonic process, the microalgal cells wall is being disrupted by shock waves from cavitation bubbles, resulting in liberation of intracellular compounds (Safafar et al., 2015; Kumari et al., 2011), so that the catalyst could penetrate easier into the cell and react with the fatty acids. It has also been reported that methanol disintegrates cell membranes more than other solvents (Safafar et al. 2015), which improves the efficiency of the transesterification for the same reason.

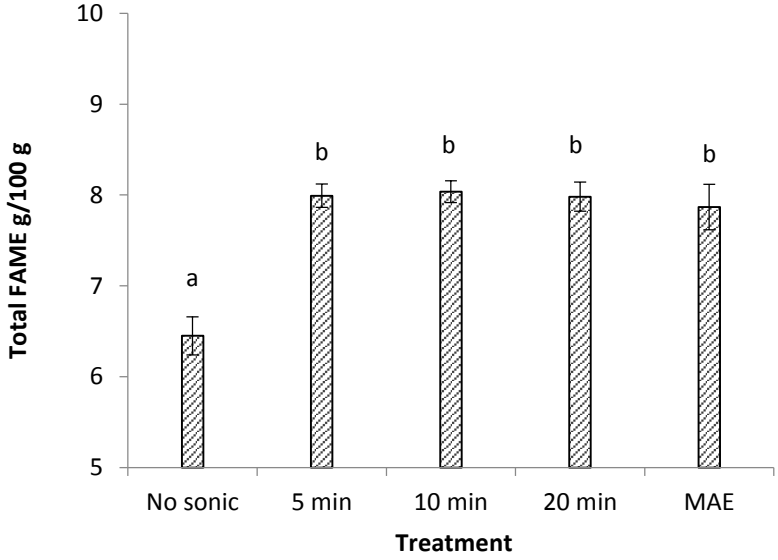

Figure 1. The effect of sonication time on the total FAME, in *Chlorella sorokiniana* (n=4). Results are shown as average values in g/100 g sample weight ±SD of two technical replicates. No sonic: sample esterified without sonication. Direct transesterification was done by procedure **A**.

On the other hand, methanol is a highly polar solvent with a relative polarity of 0.762 (Reichardt, 2003) so to improve the recovery of methyl esters, a combination of another solvent with lower polarity would enhance the process, which was already found in our preliminary studies (data not shown). For this reason heptane (polarity index of 0.06), and toluene (polarity index of 0.012) were selected for the main experiments (**Figure 1**).

The comparison of various DT procedures is shown in **Table 1**. Results of procedure **E** was omitted due to very low recovery which made the GC analysis impossible. The highest FAME content was found in the sample treated with procedure **A**, which was very

similar to the total FAME in SRM® 1946 (87.6±0.17 g/100 g sample weight) and by MAE (78.6±0.41 g/100 g sample weight). Higher FAME contents were achieved in the samples treated by procedures **A, C, F,** which contained toluene as co-solvent, compared to procedures **B** and **D** with only heptane.

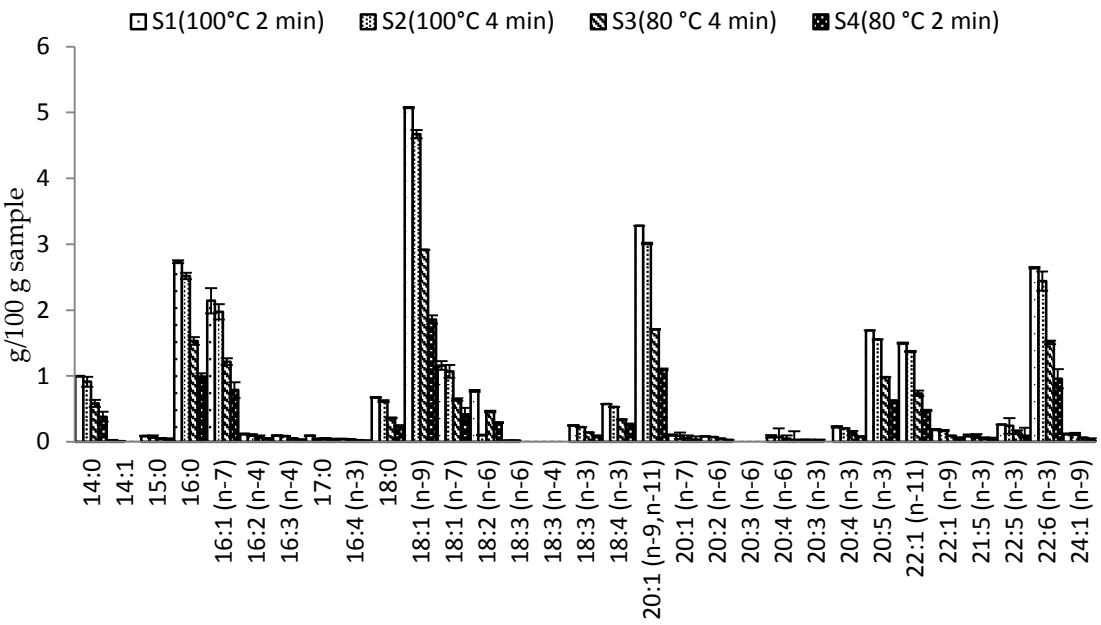

Figure 3. Effect of time and temperature of BF3 esterification on the composition and and total fatty acids extracted from fish tissue. Results are shown as average values ± SD of two technical replicates. Data are presented as g/100 g sample weight.

It has been reported that the solubility of FAME in toluene is higher than in other solvents such as heptane or chloroform. Chloroform could not be used because at the presence of methoxide, as it presumably produces dichlorocarbene, which reacts with double bonds in the structure of unsaturated fatty acids (Carrapiso and García, 2000).

Samples which were processed by microwave (C-F) for both one step and two steps DT, showed lower contents of FAME compared to those treated in a water bath. Also in comparison with one-step transesterification procedures (**E, F**) better results were achieved by the two-step procedures (**A-D**). Transesterification by basic catalysts such as NaOH and KOH is fast and efficient, and suitable for Triacylglycerol (TAG) lipids. When the sample include high contents of free fatty acids (such as in microalgae), basic catalysts could not be used for single step DT (Shantha & Ackman, 1991). Another negative issue

with basic catalysts is the saponification reactions of esters, which occurs in the presence of water. However, the saponification reaction is much slower than transesterification (Carrapiso and García, 2000). When the esterification reaction was enhanced by sonication, the time and temperature could be reduced in favour of the transesterification reaction, even in the presence of water (**Figure 2**). The additional two minutes heating at 100°C, has improved the results for procedures A and B, which could be attributed to the improvement of lipid extractability due to heat treatment, as the alkaline esterification do not requires heat treatment (Dong et al. 2015).

An acidic catalyst such as sulfuric acid and methylene chloride has mostly been used as favoured DT catalysts in microalgae biomass (Le page' and Roy, 1986). Sulfuric acid is efficient for esterification of both free fatty acids and triacylglycerols, but requires very long reaction time and high temperature for appropriate results (Brunton, 2015), and therefore it is not the best choice for a rapid and routine DT procedure. Methanolic boron trifluoride (BF3) is a commonly used catalyst for the preparation of FAME due to its high esterifying power (Carrapiso García, 2000), while toxic and unstable. The combination of methanolic alkaline-BF3 transesterification has already been used in a few studies on microbial biomass(Carrapiso García, 2000). The production of artifacts, which has been shown as the disadvantage of BF3/methanol (Carrapiso García, 2000), requires high temperature and long reaction time, so when the reaction time is low (2 minutes), it would no longer be a problem. No single derivatization procedure exists that simultaneously derivatize all different lipid classes such as triacylglycerols, free fatty acids, phospholipids, glycolipids and others (Nuerenberg et al. 2007),which are normally presents in microalgae lipid fraction, so the two-step DT may probably provides better results in the matrices such as microalgae.

The fatty acid composition was very similar, for procedure **A** and B, but the results of procedures **C, D** and F were not appropriate.

Comparison of DT with MAE for various sample types

In **Figure 4,** fatty acid composition of the SRM® 1946 (n=2) derivatized by MAE is compared to DT (procedure A). The fatty acid composition and amounts of each fatty acid were the same in both DT and MAE.

Table 1. Total recovered fatty acid methyl esters and fatty acid compositions of SRM® 1946 (n=4). Results are shown as average values ± SD of two technical replicates. Data are presented as g/100 g sample weight.

Procedure	A	B	C	D	F
FAME					
14:0	2.58±0.25	2.23±0.13	2.13±0.12	1.67±0.00	0.86±0.09
14:1	0.28±0.08	0.36±0.04	0.27±0.13	0.06±0.00	0.07±0.05
15:0	0.39±0.15	0.45±0.15	0.21±0.06	0.18±0.00	0.18±0.01
16:0	11.25±0.75	10.22±0.50	9.37±0.47	8.69±0.00	5.35±0.20
16:1 (n-7)	7.24±0.41	6.61±0.40	5.59±0.11	5.43±0.00	3.05±0.03
16:2 (n-4)	0.36±0.07	0.26±0.06	0.20±0.01	0.30±0.00	0.06±0.03
16:3 (n-4)	0.35±0.23	0.13±0.03	0.10±0.01	0.14±0.00	0.10±0.06
17:0	0.00±0.00	0.33±0.08	0.23±0.01	0.16±0.00	0.12±0.09
17:1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.07±0.05
16:4 (n-3)	0.07±0.00	0.17±0.02	0.14±0.01	0.41±0.00	0.00±0.00
18:0	2.41±0.05	2.26±0.07	2.16±0.16	2.19±0.00	1.20±0.09
18:1 (n-9)	22.15±0.55	20.52±0.93	19.03±1.02	17.37±0.50	9.27±0.01
18:1 (n-7)	3.09±0.14	2.92±0.10	2.52±0.09	2.49±0.20	1.51±0.06
18:2 (n-6)	3.10±0.11	2.94±0.10	2.51±0.08	2.48±0.10	1.32±0.07
18:2(n-4)	0.18±0.01	0.18±0.00	0.13±0.01	0.18±0.00	0.09±0.07
18:3 (n-6)	1.93±0.09	1.84±0.08	1.45±0.02	1.47±0.10	0.80±0.06
18:3 (n-4)	0.82±0.04	0.78±0.03	0.54±0.00	0.59±0.00	0.33±0.06
18:3 (n-3)	0.06±0.00	0.06±0.01	0.07±0.04	0.04±0.00	0.00±0.00
20:0	0.00±0.00	0.00±0.00	0.00±0.00	0.08±0.01	0.04±0.06
20:1 (n-9, n-11)	1.28±0.04	1.20±0.05	1.15±0.08	0.99±0.01	0.76±0.06
20:1 (n-7)	0.42±0.09	0.39±0.01	0.38±0.00	0.30±0.00	0.18±0.03
20:2 (n-6)	0.92±0.01	0.86±0.03	0.46±0.52	0.75±0.01	0.39±0.09
20:3 (n-6)	0.46±1	0.43±0.02	0.58±0.17	0.30±0.00	0.19±0.04
20:4 (n-6)	2.02±0.02	1.95±0.09	1.55±0.02	1.44±0.03	0.93±0.02
20:3 (n-3)	0.82±0.14	0.77±0.01	0.69±0.03	0.53±0.00	0.30±0.01
20:4 (n-3)	2.96±0.06	2.77±0.12	2.28±0.05	2.13±0.04	1.23±0.07
20:5 (n-3)	2.46±0.09	2.36±0.11	1.70±0.01	1.78±0.06	1.12±0.03
22:1 (n-11)	0.45±0.40	0.16±0.01	0.17±0.01	0.00±0.00	0.05±0.02
22:1 (n-9)	0.13±0.12	0.04±0.00	0.04±0.00	0.00±0.00	0.06±0.09
21:5 (n-3)	0.18±0.01	0.17±0.01	0.12±0.00	0.83±0.00	0.11±0.10
722:5 (n-3)	2.73±0.05	2.56±0.10	2.14±0.06	2.06±0.08	1.17±0.08
22:6 (n-3)	7.41±0.21	7.16±0.29	5.39±0.09	5.69±0.09	3.64±0.02
24:1 (n-9)	0.21±0.22	0.32±0.02	0.03±0.01	0.00±0.00	0.24±0.03
Sum	78.9±0.83	73.4±1.18	63.3±2.15	60.7±0.46	34.8±2.44

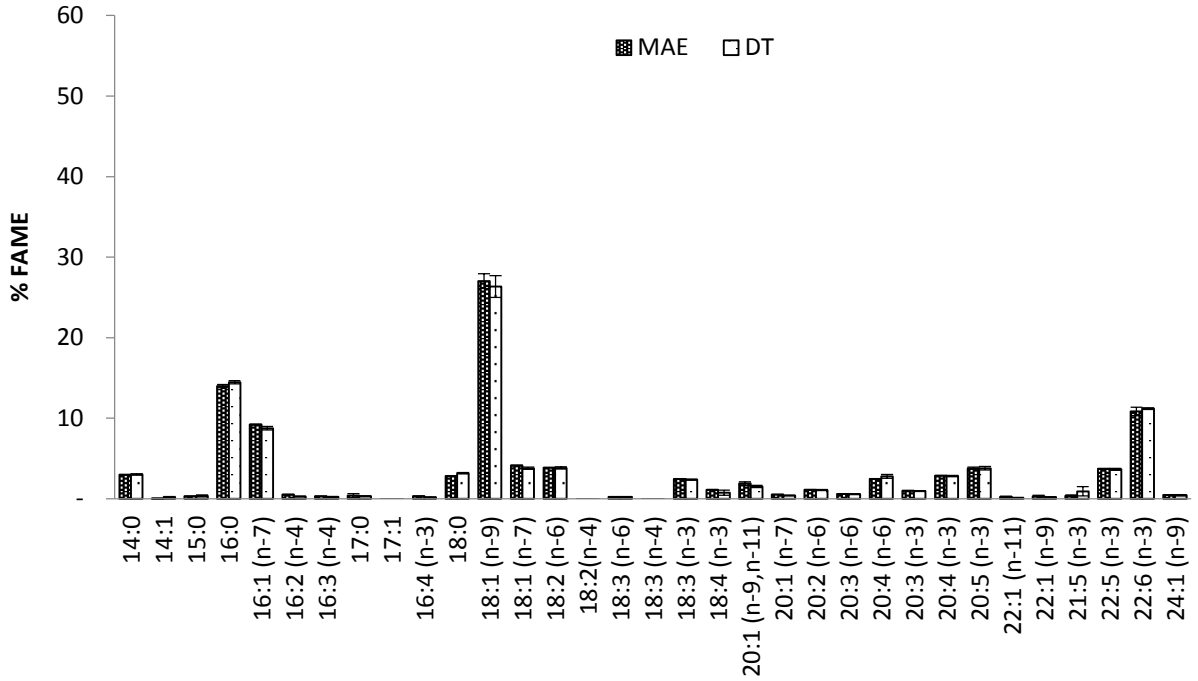

Figure 4. Comparison of the fatty acid composition of SRM® 1946 (n=4) derivatized by MAE and DT. Results are shown as average values± SD as % of each fatty acid per total fatty acids. Direct transesterification was done by procedure **A**.

In order to evaluate the selected procedure on microalgae, six biomass samples from different species were analysed by DT and the results compared to MAE. In addition to microalgae, samples of krill meal (high levels of phospholipids) and salmon fish tissue were also analysed. Results were virtually identical to the FAME by MAE ($R^2 = 0.99$) as the standard reference method (**Figure 5**).

Some examples of fatty acid compositions by MAE and DT are demonstrated in **Figure 6.** The best results were observed for microalgae with lipid contents more than or equal to 8.0% DW, while for samples with lipids less than or equals to 2% DW, differences in fatty acid content were higher than 10 % for major fatty acids. The optimum range of sample weight was evaluated to be 50-100 mg, which equals to a sample to reagent ratio of 1:10 to 1:20. When the sample weight was either lower than 50 mg, or more than 100 mg the variations increased to the values more than 10%.

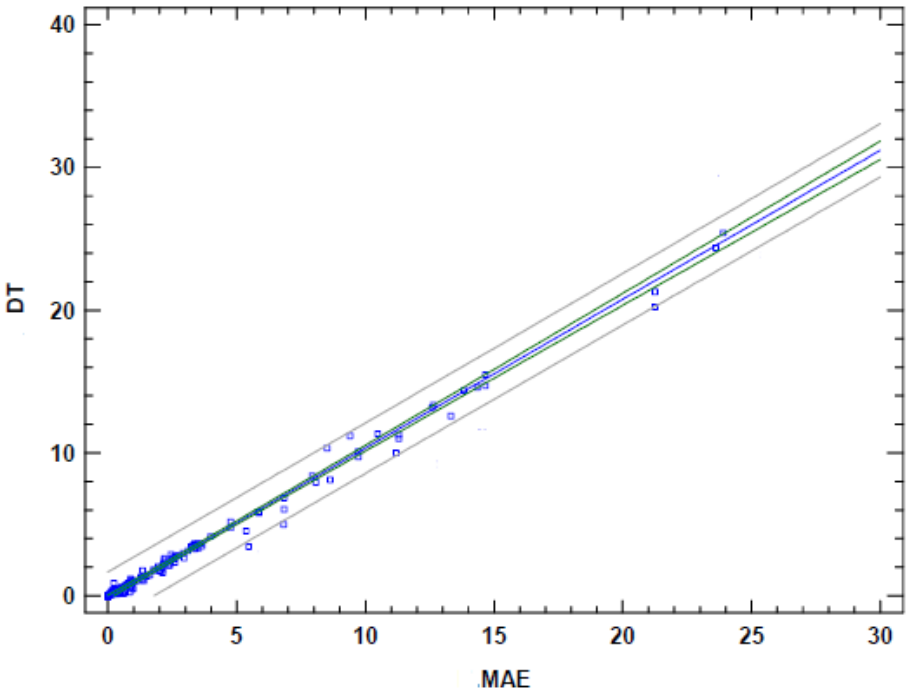

Figure 5. Correlation of the fatty acids data (with each fatty acids as a data point) obtained from six species of microalgae, krill meal and salmon flesh between DT and MAE (R-squared = 99.0 %, α=0.05%, and n=10), Procedure A was used as DT method.

To our knowledge, two step DT by alkaline and BF3 catalysts was not previously applied to microalgae. However, Park and Goins (1994) presented a similar two-step DT, which was successfully tested on various lipid containing food products (including rainbow trout). The method included heating at 90°C for 10 min for the methanolysis by 0.5 N methanolic sodium hydroxide followed by heating at 90°C for another 10 min after addition of 14% BF- methanol. This procedure was reported as suitable for all food samples, and particularly phospholipid-rich foods. The FAME profile was also reported as identical to the reference method. It should be considered that in microalgae, in addition to the presence of different lipid classes(phospholipids, glycolipids, free fatty acids and triacylglycerols) the cell wall structures and strength are significantly different (Dong et al.,2015). A suitable DT method should be able to extract and treat lipids entrapped inside of cell wall matrices.

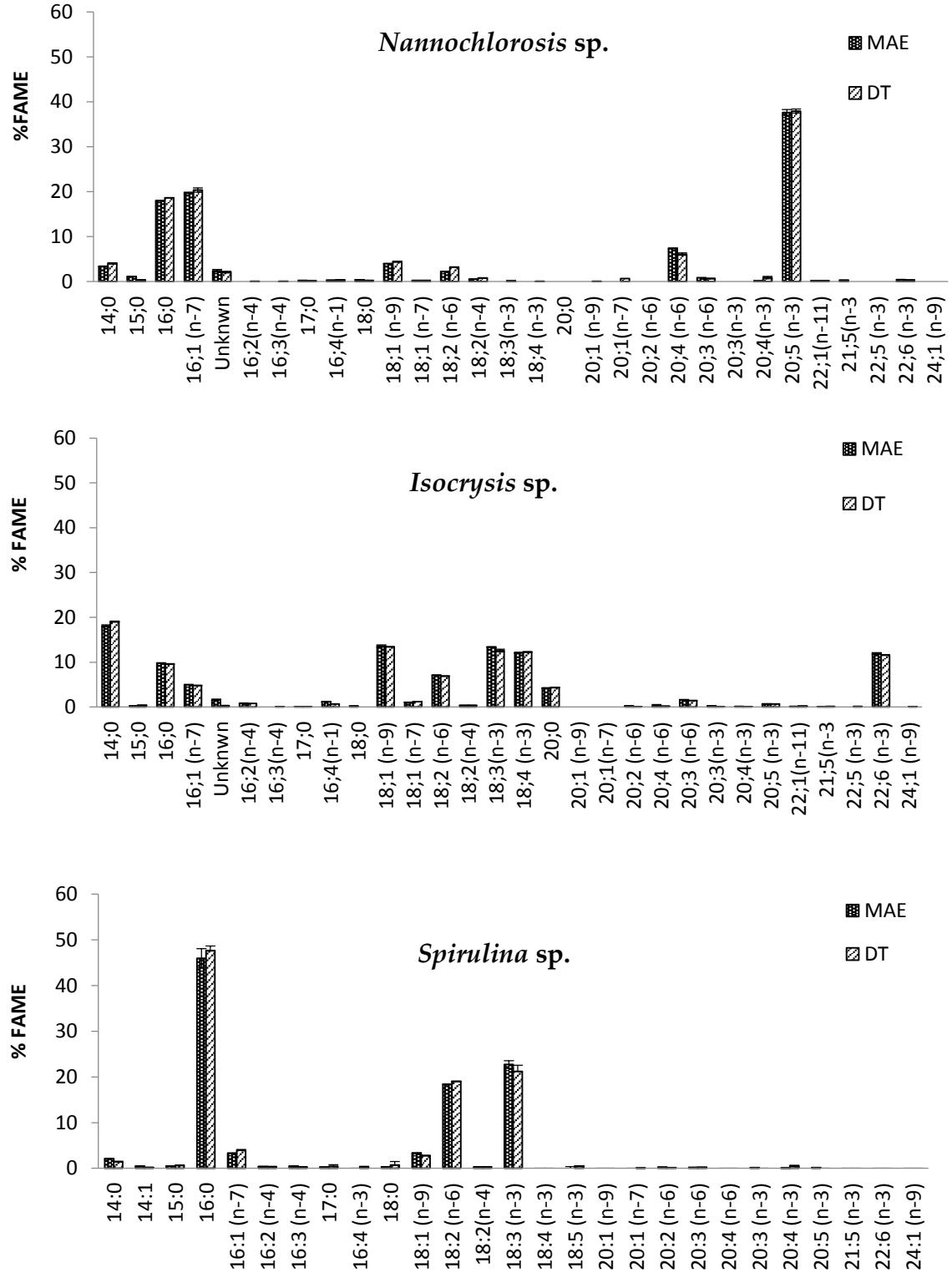

Figure 6. The fatty acid composition of some microalgae biomass derivatized by DT and MAE. (n=2). Results are shown as average values± SD as normalised % of each fatty acid per total fatty acids. Direct transesterification(DT) was done by procedure **A**.

The DT method demonstrated in this paper is fast and accurate and could comparatively substitute time-consuming standard procedure which includes B&D extraction followed by transesterification. The time required for the DT was only 14 minutes. Hence, the

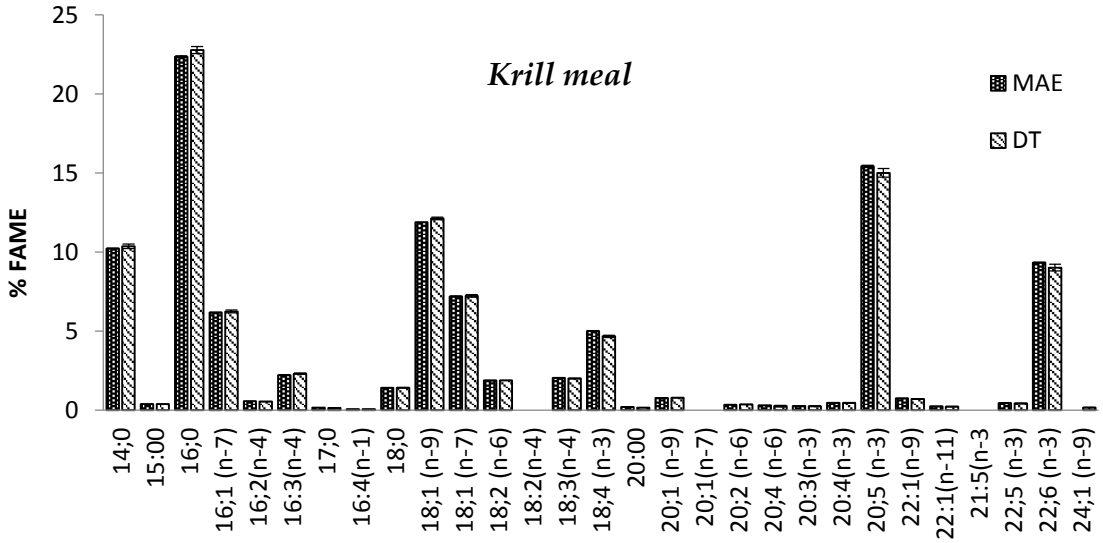

Figure 7. The fatty acid composition of Krill meal derivatized by DT and MAE. (n=2). Results are shown as average values± SD as normalised % of each fatty acid per total fatty acids. Direct transesterification(DT) was done by procedure **A.**

demonstrated method is the fastest DT method ever reported. The gas chromatographic separation-identification procedure already requires very short time (12 minutes), which decreases the total analysis time to less than 30 minutes.

Conclusions

The DT method omitted lengthy lipid extractions. FAME were prepared directly from base-catalyzed methanolysis followed by BF3 methylation reactions. The GC analysis of DT-FAME revealed fatty acid composition virtually identical to that of FAME by the standard method. The DT method could be suggested as a rapid and routine control measure to verify the quality and/or purity of microalgae biomass during cultivation or application as a food/feed ingredient.

Founding

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Compliance with Ethical Standards

Informed Consent

Not applicable.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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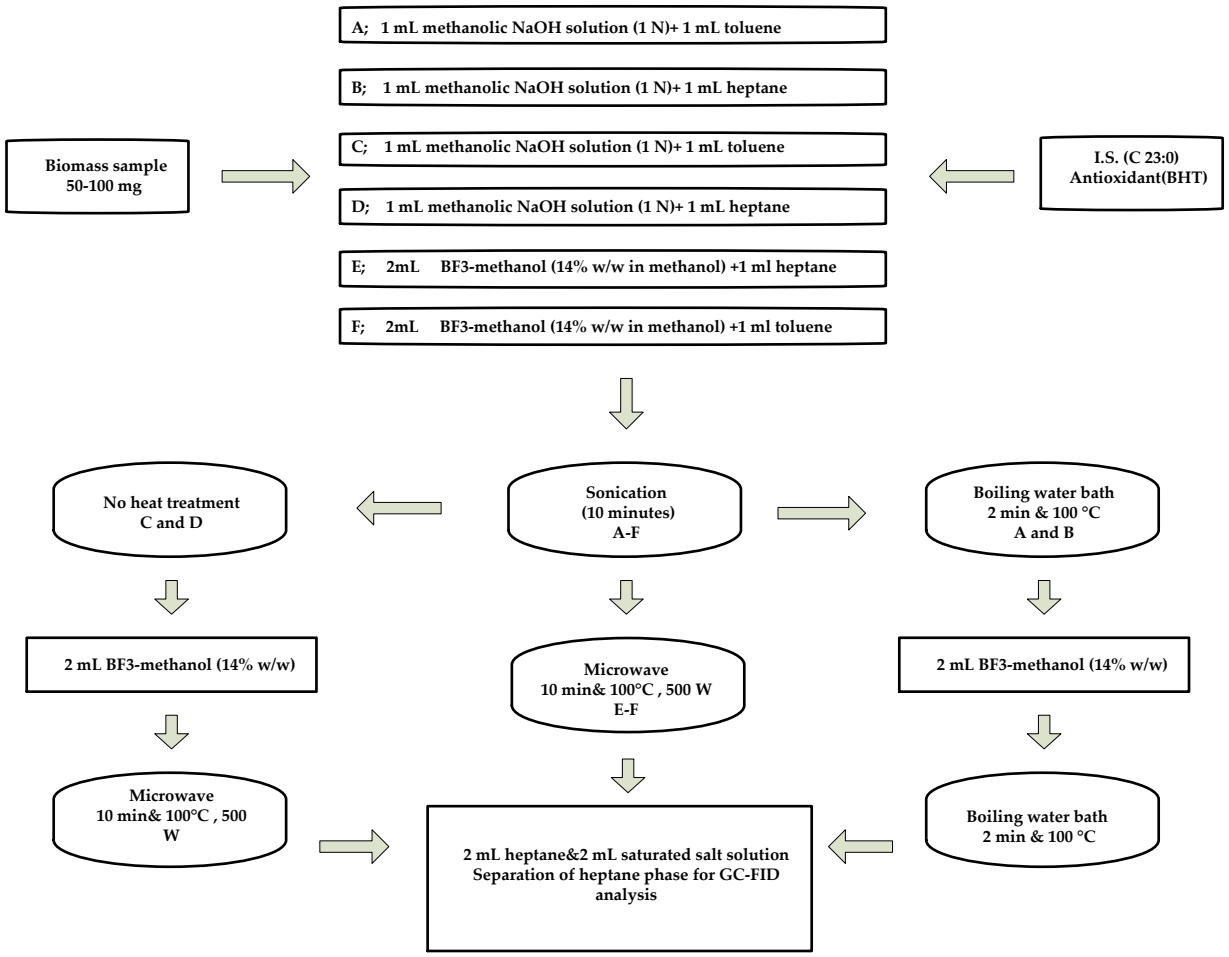

Figure 1. The experimental design for *in situ* transesterification of the fish tissue biomass. For all of the procedures, the SRM® 1946 was used. Procedures E and F treated by the single step microwave assisted esterification using just BF3(14% in methanol) as catalyst. In Procedures A, C and F, toluene was used in the initial mix, while for other (B, D and E), heptane was added instead. All samples were sonicated for 10 minutes, after addition of internal standard and antioxidant. Samples A and b were heated for 2 minutes at 100℃, and in a boiling water bath, before and after addition of BF3 (14% in methanol), while samples C and D esterified in microwave after addition of methanolic BF3.

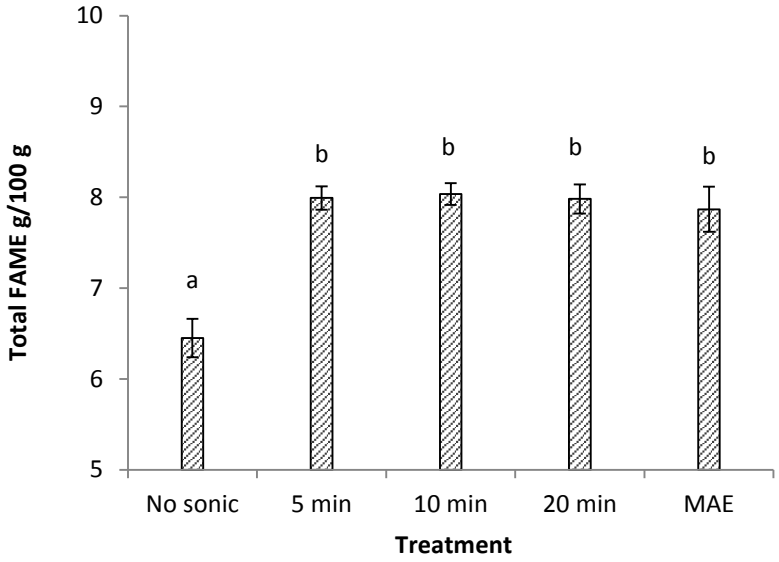

Figure 2. The effect of sonication time on the total FAME, in *Chlorella sorokiniana* (n=4). Results are shown as average values in g/100 g sample weight ±SD of two technical replicates. No sonic: sample esterified without sonication. Direct transesterification was done by procedure **A**.

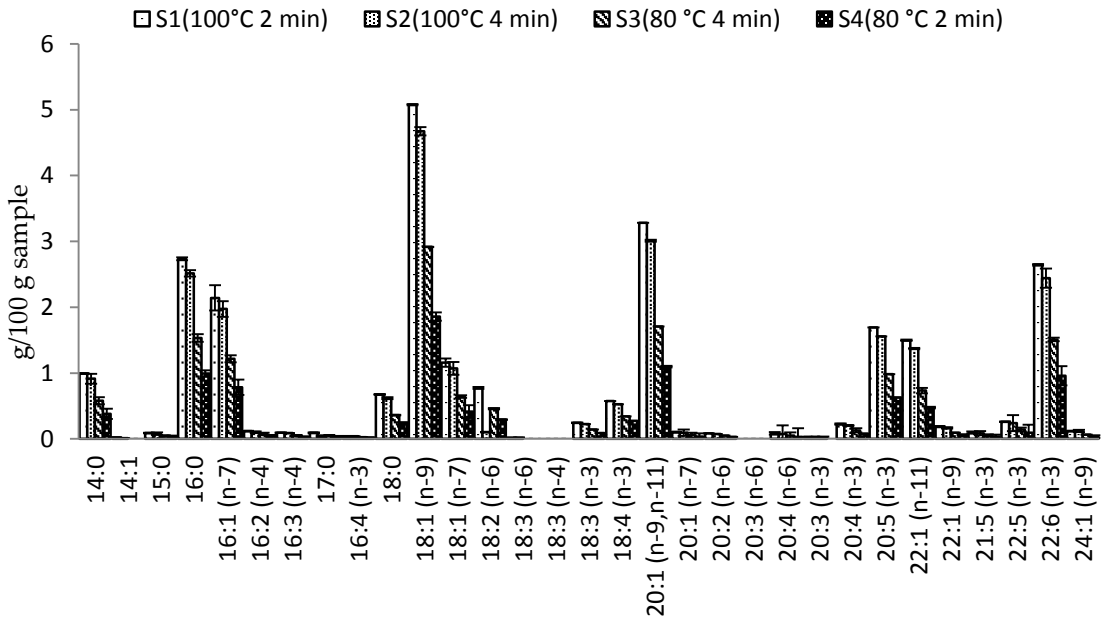

Figure 3. Effect of time and temperature of BF3 esterification on the composition and and total fatty acids extracted from fish tissue. Results are shown as average values ± SD of two technical replicates. Data are presented as g/100 g sample weight.

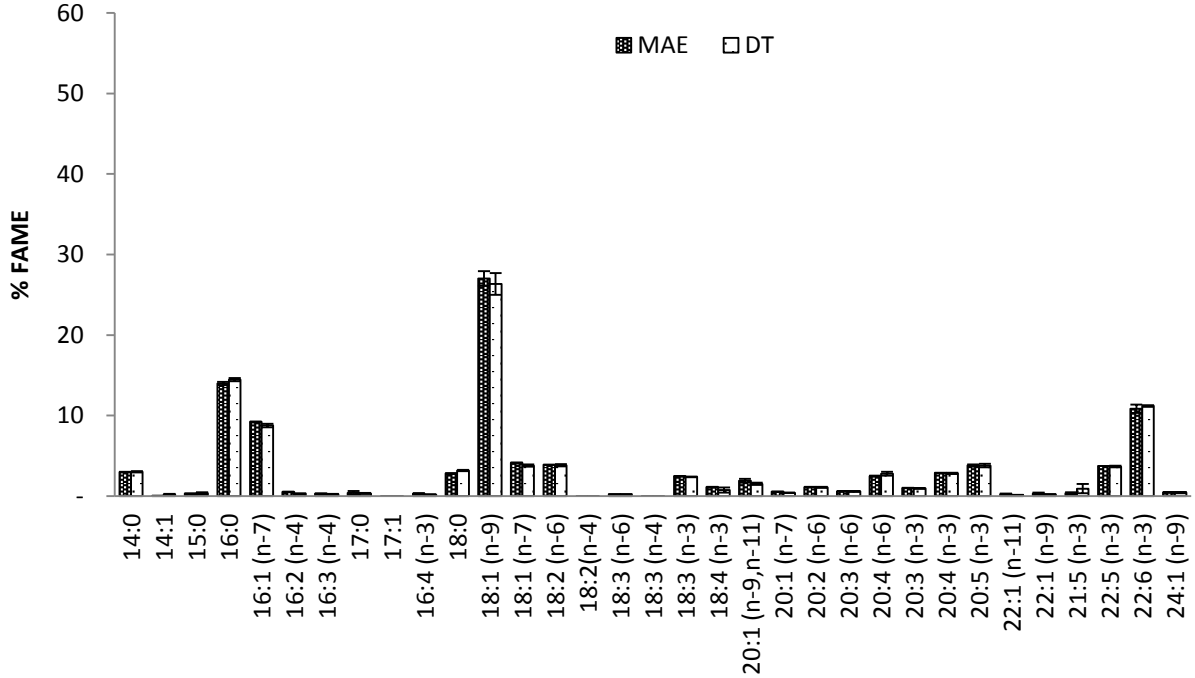

Figure 4. Comparison of the fatty acid composition of SRM® 1946 (n=4) derivatized by MAE and DT. Results are shown as average values± SD as % of each fatty acid per total fatty acids. Direct transesterification was done by procedure **A**.

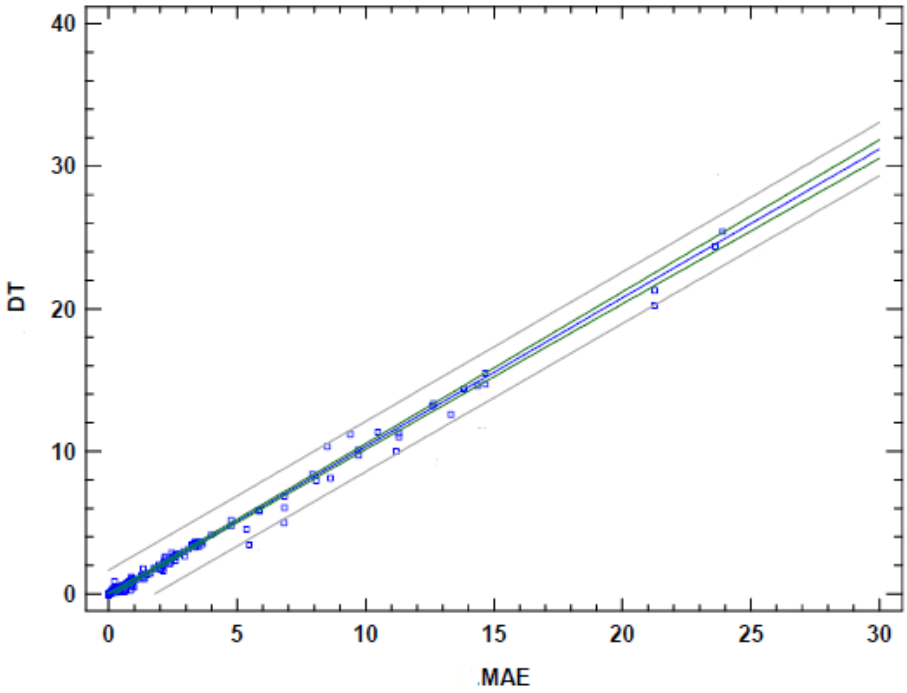

Figure 5. Correlation of the fatty acids data (with each fatty acids as a data point) obtained from six species of microalgae, krill meal and salmon flesh between DT and MAE (R-squared = 99.0 %, α=0.05%, and n=10), Procedure A was used as DT method.

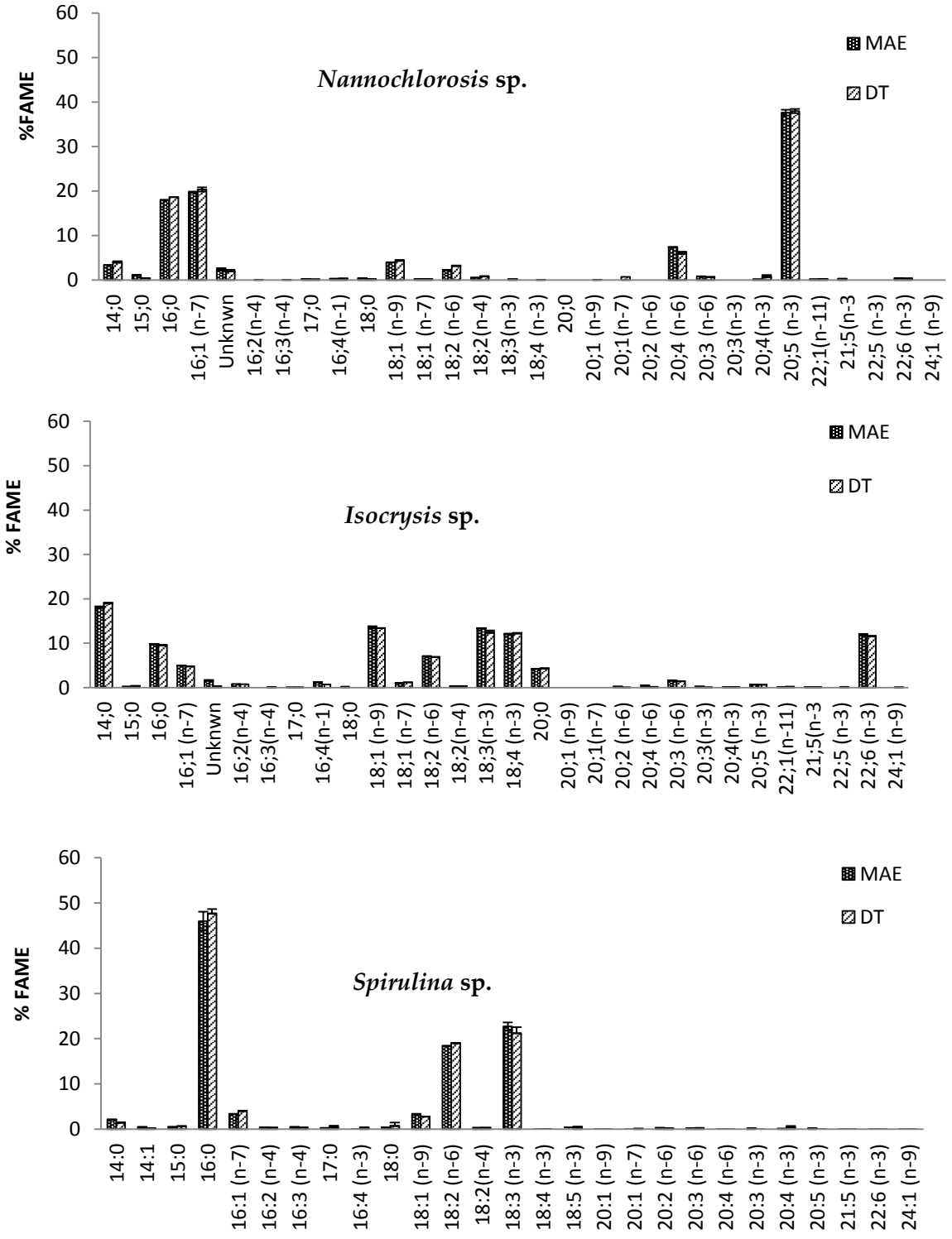

Figure 6. The fatty acid composition of some microalgae biomass derivatized by DT and MAE. (n=2). Results are shown as average values± SD as normalised % of each fatty acid per total fatty acids. Direct transesterification(DT) was done by procedure **A**.

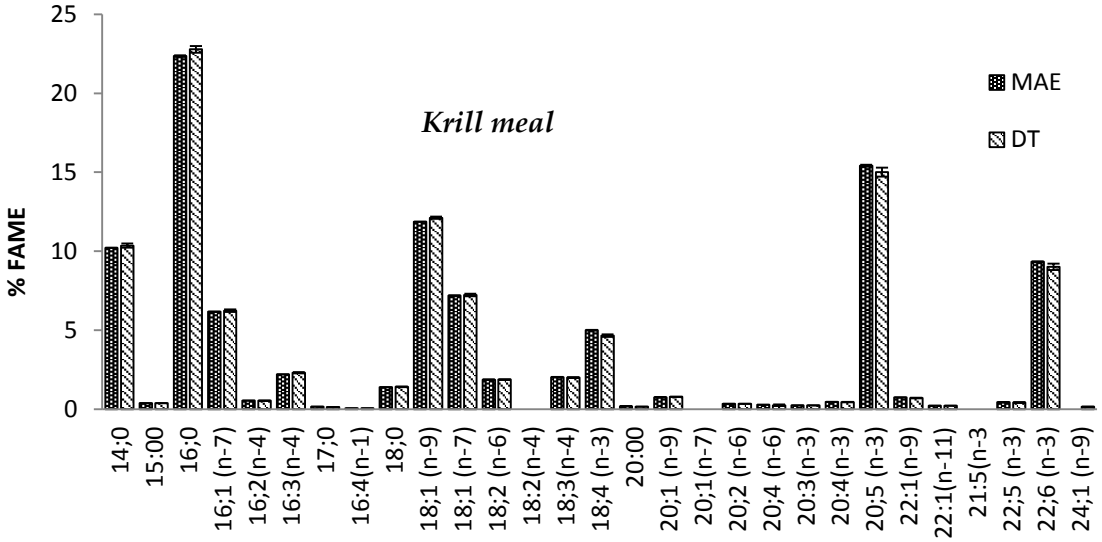

Figure 7. The fatty acid composition of Krill meal derivatized by DT and MAE. (n=2). Results are shown as average values± SD as normalised % of each fatty acid per total fatty acids. Direct transesterification(DT) was done by procedure **A.**

Table 1. Total recovered fatty acid methyl esters and fatty acid compositions of SRM® 1946 (n=4). Results are shown as average values ± SD of two technical replicates. Data are presented as g/100 g sample weight.

Procedure FAME	A	B	C	D	F
14:0	2.58±0.25	2.23±0.13	2.13±0.12	1.67±0.00	0.86±0.09
14:1	0.28±0.08	0.36±0.04	0.27±0.13	0.06±0.00	0.07±0.05
15:0	0.39±0.15	0.45±0.15	0.21±0.06	0.18±0.00	0.18±0.01
16:0	11.25±0.75	10.22±0.50	9.37±0.47	8.69±0.00	5.35±0.20
16:1 (n-7)	7.24±0.41	6.61±0.40	5.59±0.11	5.43±0.00	3.05±0.03
16:2 (n-4)	0.36±0.07	0.26±0.06	0.20±0.01	0.30±0.00	0.06±0.03
16:3 (n-4)	0.35±0.23	0.13±0.03	0.10±0.01	0.14±0.00	0.10±0.06
17:0	0.00±0.00	0.33±0.08	0.23±0.01	0.16±0.00	0.12±0.09
17:1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.07±0.05
16:4 (n-3)	0.07±0.00	0.17±0.02	0.14±0.01	0.41±0.00	0.00±0.00
18:0	2.41±0.05	2.26±0.07	2.16±0.16	2.19±0.00	1.20±0.09
18:1 (n-9)	22.15±0.55	20.52±0.93	19.03±1.02	17.37±0.50	9.27±0.01
18:1 (n-7)	3.09±0.14	2.92±0.10	2.52±0.09	2.49±0.20	1.51±0.06
18:2 (n-6)	3.10±0.11	2.94±0.10	2.51±0.08	2.48±0.10	1.32±0.07
18:2(n-4)	0.18±0.01	0.18±0.00	0.13±0.01	0.18±0.00	0.09±0.07
18:3 (n-6)	1.93±0.09	1.84±0.08	1.45±0.02	1.47±0.10	0.80±0.06
18:3 (n-4)	0.82±0.04	0.78±0.03	0.54±0.00	0.59±0.00	0.33±0.06
18:3 (n-3)	0.06±0.00	0.06±0.01	0.07±0.04	0.04±0.00	0.00±0.00
20:0	0.00±0.00	0.00±0.00	0.00±0.00	0.08±0.01	0.04±0.06
20:1 (n-9, n-11)	1.28±0.04	1.20±0.05	1.15±0.08	0.99±0.01	0.76±0.06
20:1 (n-7)	0.42±0.09	0.39±0.01	0.38±0.00	0.30±0.00	0.18±0.03
20:2 (n-6)	0.92±0.01	0.86±0.03	0.46±0.52	0.75±0.01	0.39±0.09
20:3 (n-6)	0.46±1	0.43±0.02	0.58±0.17	0.30±0.00	0.19±0.04
20:4 (n-6)	2.02±0.02	1.95±0.09	1.55±0.02	1.44±0.03	0.93±0.02
20:3 (n-3)	0.82±0.14	0.77±0.01	0.69±0.03	0.53±0.00	0.30±0.01
20:4 (n-3)	2.96±0.06	2.77±0.12	2.28±0.05	2.13±0.04	1.23±0.07
20:5 (n-3)	2.46±0.09	2.36±0.11	1.70±0.01	1.78±0.06	1.12±0.03
22:1 (n-11)	0.45±0.40	0.16±0.01	0.17±0.01	0.00±0.00	0.05±0.02
22:1 (n-9)	0.13±0.12	0.04±0.00	0.04±0.00	0.00±0.00	0.06±0.09
21:5 (n-3)	0.18±0.01	0.17±0.01	0.12±0.00	0.83±0.00	0.11±0.10
722:5 (n-3)	2.73±0.05	2.56±0.10	2.14±0.06	2.06±0.08	1.17±0.08
22:6 (n-3)	7.41±0.21	7.16±0.29	5.39±0.09	5.69±0.09	3.64±0.02
24:1 (n-9)	0.21±0.22	0.32±0.02	0.03±0.01	0.00±0.00	0.24±0.03
Sum	78.9±0.83	73.4±1.18	63.3±2.15	60.7±0.46	34.8±2.44

Paper 3

High-EPA Biomass from *Nannochloropsis salina* Cultivated in a Flat-Panel Photo-Bioreactor on a Process Water-Enriched Growth Medium.

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High-EPA Biomass from *Nannochloropsis salina* Cultivated in a Flat-Panel Photo-Bioreactor on a Process Water-Enriched Growth Medium

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Abstract: *Nannochloropsis salina* was grown on a mixture of standard growth media and pre-gasified industrial process water representing effluent from a local biogas plant. The study aimed to investigate the effects of enriched growth media and cultivation time on nutritional composition of *Nannochloropsis salina* biomass, with a focus on eicosapentaenoic acid (EPA). Variations in fatty acid composition, lipids, protein, amino acids, tocopherols and pigments were studied and results compared to algae cultivated on F/2 media as reference. Mixed growth media and process water enhanced the nutritional quality of *Nannochloropsis salina* in laboratory scale when compared to algae cultivated in standard F/2 medium. Data from laboratory scale translated to the large scale using a 4000 L flat panel photo-bioreactor system. The algae growth rate in winter conditions in Denmark was slow, but results revealed that large-scale cultivation of *Nannochloropsis salina* at these conditions could improve the nutritional properties such as EPA, tocopherol, protein and carotenoids compared to laboratory-scale cultivated microalgae. EPA reached 44.2% $\pm$ 2.30% of total fatty acids, and α-tocopherol reached 431 $\pm$ 28 µg/g of biomass dry weight after 21 days of cultivation. Variations in chemical compositions of *Nannochloropsis salina* were studied during the course of cultivation. *Nannochloropsis salina* can be presented as a good candidate for winter time cultivation in Denmark. The resulting biomass is a rich source of EPA and also a good source of protein (amino acids), tocopherols and carotenoids for potential use in aquaculture feed industry.

Keywords: EPA; *Nannochloropsis salina*; industrial process water; microalgae; carotenoids; amino acid; tocopherol; large scale; flat panel photo-bioreactor

1. Introduction

Microalgae are autotrophic microorganisms, which are able to produce biomass from solar energy, CO_2 and nutrients, with higher photosynthetic activity compared to terrestrial plants [1,2]. Resulting biomass includes important metabolites such as carbohydrates, lipids, proteins and also many other bioactive compounds like pigments and phenolics. Microalgal biomass may be used for different applications, such as biofuel production, wastewater treatment, production and extraction of bioactive compounds, or as a food/feed ingredient [3,4]. Actually, one of the most promising applications of microalgae biomass is as feed for aquatic animals. Algae are utilized for various applications in aquaculture. The importance of microalgae in aquaculture food chains is mainly due to their fatty acid, carotenoids and protein (amino acid) composition [5,6]. Use of microalgae as a rich source of fatty acids for the aquaculture has become the focus of industrial and scientific developments. Microalgae suitable for utilization in aquaculture should possess certain important characteristics such as appropriate

nutritional value, high production rates, suitability for mass cultivation, resistance to fluctuating growth conditions, and nontoxicity [7]. Species such as *Chlorella, Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, and *Thalassiosira* are reported as the most frequent used [8].

Nannochloropsis is a genus comprised of small (less than 5 μm) coccoid uni cells and is known primarily from the marine environment [9]. Different species from the genus *Nannochloropsis* have received sustained interest due to their high biomass and lipid productivity and their potential for cultivation at a large scale [9]. Members of the genus *Nannochloropsis* (Eustigmatophyceae) are widely distributed in marine and fresh water ecosystems; and individually in coastal waters [9]. There are six recognized species in the *Nannochloropsis* genus as *Nannochloropsis gaditana*, *Nannochloropsis salina*, *Nannochloropsis granulata*, *Nannochloropsis limnetica*, *Nannochloropsis oceanica*, and *Nannochloropsis oculata* [9,10].

The microalga *Nannochloropsis salina* is one of the most promising candidates for large scale cultivation. On the other hand this microalga accumulate high amounts of eicosapentaenoic acid (EPA), which is one of the most favored fatty acids in aquaculture [11]. Several scientific investigations have been published regarding the biotechnological potential of *Nannochloropsis salina* [12–16]. Feasibility of large scale cultivation of *Nannochloropsis* sp. in photo bioreactors has been studied in several studies [16–19]. Zittelli et al. [17] cultivated *Nannochloropsis* sp. in a flat panel photo-bioreactor under artificial light and demonstrated that higher illumination increased the biomass productivity but decreased the content of pigments (Chlorophyll a and carotenoids). Variations in lipid classes and fatty acid composition were studied during 2007–2009 in *Nannochloropsis oculata* cultivated in vertical flat panel photo-bioreactors and explained by seasonal light and temperature [18].

The effect of light-path length in flat plate reactors on eicosapentaenoic acid, lipid and biomass productivity of outdoor cultivated *Nannochloropsis* sp. was tested by Zou et al. [19]. In this study, the optimal light-path for culturing *Nannochloropsis* was reported as ca. 10 cm. Olofsen et al. [20] investigated lipid and biomass productivity and fatty acid composition of *Nannochloropsis oculata* cultivated in flat panel photo-bioreactors under different nitrogen limitation conditions during autumn and spring. In this study, *Nannochloropsis occulata* demonstrated high biomass productivity under nitrogen limitation comparing to nitrogen starvation during both seasons. Eicosapentaenoic acid (EPA) production by *Nannochloropsis gaditana* in different pilot scale outdoor photo-bioreactors was investigated by Camacho-Rodríguez et al. [21]. In this study, the biomass productivity in a vertical flat-panel bioreactor (FP-PBR) was reported as 10-fold higher compared to the traditional production system. San Pedro et al. [22] studied the feasibility of growing *Nannochloropsis gaditana* in outdoors raceway ponds. In this study, growth and lipid production models in relation to operational and environmental conditions were also developed.

Large scale production of microalgae biomass faces several challenges to be economically feasible, and the cultivation cost is one of the major factors affecting the price of final product. One of the strategies to resolve this problem is to use a low-cost culture medium such as waste water [23].

Some species of microalgae are capable of growing in municipal, industrial and agricultural wastewater, producing biomass while reducing the load of nitrogen and phosphorus [24–26]. On the other hand, the wastewater composition should be free of pollutants such as heavy metals, when the resulting microalgal biomass is intended to be used as a feed ingredient. Trace metals at levels that do not inhibit algal growth still cause problems via their presence in algae biomass are used in aquaculture or for animal feedstocks [25]. Under anaerobic conditions, methanogenic conversion of organic matter to biogas will result in methane, CO_2, and an effluent which contains ammonia. The conversion process, known as anaerobic digestion, is being done in an anaerobic sludge tower reactor with internal circulation (ICT), so the effluent is called IC water (ICW). The performance of a microalgae cultivated on ICW effluent depends on a variety of factors including characteristics of algal species, properties of the anaerobic digestion effluent, and operational parameters during algal growth [25,26].

The objective of the present study was to find an economically viable use of the industrial process water nutrients and to investigate the effects of ICW enriched growth media and cultivation time on nutritional composition of *Nannochloropsis salina* biomass, with a focus on eicosapentaenoic acid (EPA). The aim was to obtain an algal biomass, which potentially could be used as an ingredient for aquaculture feed. The reference standard culture medium used was the F/2 medium [27], which is widely used as enriched seawater medium for growing marine microalgae. The F/2 medium has a low concentration of NO_3^- as nitrogen source (0.75 mM), so addition of another N source (ammonium) affects growth rate and chemical composition of resulting biomass. The optimal growth condition was determined via batch laboratory experiments in order to provide basic reference data to be used in large scale. The optimal growth conditions were subsequently validated in large scale. This study revealed changes in nutritional composition of biomass during the course of cultivation and demonstrated which harvest time would be the optimal.

2. Results and Discussion

2.1. Growth Rate in Laboratory Scale Experiments

A number of nitrogen compounds are available for algae to assimilate: nitrate, nitrite, ammonium, and even organic nitrogen, and in general ammonium is the most preferable chemical form because it takes less energy for algae to assimilate into amino acids [28]. On the other hand, ammonium tolerance is different for various algae species and can be strongly influenced by the environmental conditions. Since free ammonia is toxic to algae, it can be stored in the system through an ammonia binding reaction [29]. In Figure 1a the effect of different levels of ICW on the growth rates of *Nannochloropsis salina* are shown. These observations showed that substitution of more than 20% of standard F/2 could retard the growth rate of *Nannochloropsis salina* at pH 8 and room temperature. Our preliminary observations (data not shown) revealed that *Nannochloropsis salina* cannot grow successfully on ICW as the main nutrient source. The nitrogen source in F/2 media is nitrate, but in process water mostly includes ammonia + ammonium-N (Table 1). The nitrogen-to-phosphorus (N/P) ratio was 15 for F/2 and 17.2 for process water, which were close to the ideal atomic ratio of 16 for *N. salina* [30]. Some previous studies reported that *Nannochloropsis* sp. can utilize ammonium as well as nitrate [14,24,25,28–31]. The growth interruption of *Nannochloropsis salina* on ICW as main nutrient source might be related to different factors including concentration of ammonia in growth media (Table 2). Ammonia toxicity is mainly attributed to NH_3 at pH > 9 and at pH < 8, toxicity is likely associated with the ammonium ion rather than ammonia. During growth of algae on a growth media containing ammonium, the pH decreases, as H^+ ions released to the medium. In contrast, growth on nitrate causes an increase in the pH due to the release of OH^- ions [31]. This phenomenon can result in less available carbon, as the main source of the carbon was CO_2, which was injected automatically when the pH raised over set value (e.g., pH = 8). Cultivation of *Nannochloropsis salina* at various pH values (8.3 ± 0.2, 7.3 ± 0.2 and 6.3 ± 0.2) with the mixed nutrient composition (20% ICW + 80% F/2) showed that at pH 6.3, the growth rate was significantly lower than at higher pH (Figure 1b). It has also been reported previously that optimum pH for *Nannochloropsis salina* ranged from 7.5 to 8.5 [32]. In this study pH 8.3 ± 0.2 was selected as the optimum pH for further experiments.

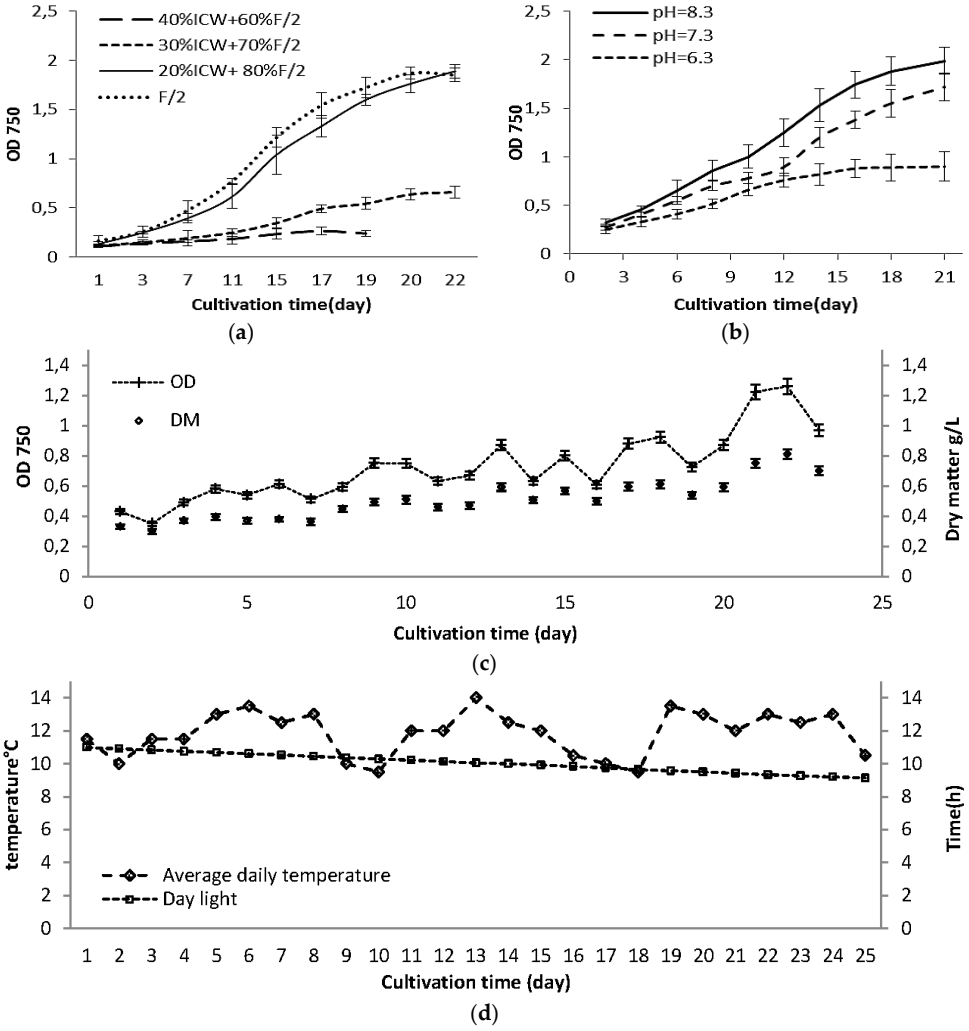


Figure 1. Growth curves for: (**a**) Effect of different levels of industrial process water on growth of *Nannochloropsis salina* at pH = 8.5; (**b**) *Nannochloropsis salina* cultivated on 20% ICW + 80% F/2 at 3 different pH; (**c**) large scale cultivated *Nannochloropsis salina*; and (**d**) variation in daily light hours and temperature during large scale cultivation. Large scale cultivation was done at pH = 8.3 ± 0.2 using 20% ICW + 80% F/2 as growth media at October–November 2015 in Kalundborg, Denmark. The standard errors are presented as bars (n = 2).

Table 1. Chemical composition of industrial wastewater.

Item	Unit	Amount
pH	-	8.1
Suspended solids	mg/L	20
Total N	mg/L	190
Ammonia + ammonium-N	mg/L	150
Nitrite + nitrate	mg/L	<0.1
Total P	mg/L	11
Sulphate	mg/L	3.6
Total Alkalinity	mmol/L	62.5
EDTA	mg/L	<0.5
Sodium(Na)	mg/L	1500
Copper (Cu)	µg/L	3.4
Iron (Fe)	mg/l	0.23

Table 2. Type and amounts of nitrogen in each growth media.

Growth Media *	NO_3^-	NH_4^+	Total N	Total P
F/2	75	-	75	5.0
20% ICW + 80% F/2	60	30	90	6.2
30% ICW + 70% F/2	52	45	97	6.8
40% ICW + 60% F/2	45	60	105	7.7

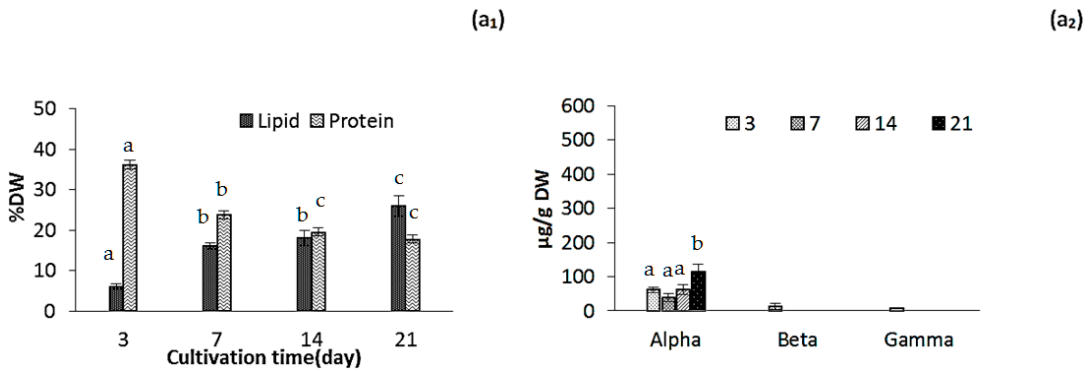
* All values are in mg/L.

2.2. Changes in Biomass Composition in Response to Growth Media

2.2.1. Protein, Lipids and Tocopherols

Lipid content increased during the cultivation from 7.9% ± 0.50% DW to 29.0% ± 1.50% DW and from 6.15% ± 1% DW to 26.0% ± 1.75% DW, for ICW + F/2 and F/2 cultivated microalgae, respectively (Figure 2a,b). Effect of growth media was evaluated as not significant while cultivation time significantly affected the oil accumulation in both experiments ($p < 0.05$). Amounts of lipids in biomass depend on several factors such as nutrient composition and environmental factors, so comparison of the results with other studies is not possible. Various amounts of lipids in *N. salina* were previously reported [11,12,14,24,33]. One study reported that under nitrate limitation, total fatty acids could exceed 70% of the biological dry mass in this specie [11], while in another study lipid accumulation in *N. salina* was the lowest (36.95% ± 0.91% DW) among 9 different *Nannochloropsis* species [12]. Several studies demonstrated that accumulation of lipids is enhanced in nitrogen-starved or deprived cultures of microalgae [11,14,32]. In our study, relatively high N/P ratio of the growth media may have impacted the lipid contents. However, our findings indicated that nitrogen starvation most likely had not occurred during cultivation for 21 days.

In general, microalgae have a limited ability to produce nitrogen storage materials when growing under nitrogen-sufficient conditions, with some exceptions in cyanobacteria. When microalgae are grown under nitrogen starvation, the most striking effect is the active and specific degradation of phycobilisomes [32]. Until available nitrogen in cell falls below a threshold value, photosynthesis still continues at lower rate. Under these circumstances, photosynthetically fixed carbon is then diverted from the protein synthesis into the pathways for carbohydrate and lipid synthesis [32], resulting in gradual decrease of protein during the cultivation. Our results confirm this phenomenon as protein content decreased during the cultivation from 39.7% DW to 18.1% DW and from 36.2% DW to 17.8% DW for ICW + F/2 and F/2 cultivated microalgae, respectively (Figure 2a_1,b_1). Both growth media and cultivation time had a statistically significant effect on protein content at the 95.0% confidence level ($p < 0.05$). Difference in protein contents was, however, not significant after day 14.



(**a**)

Figure 2. *Cont.*

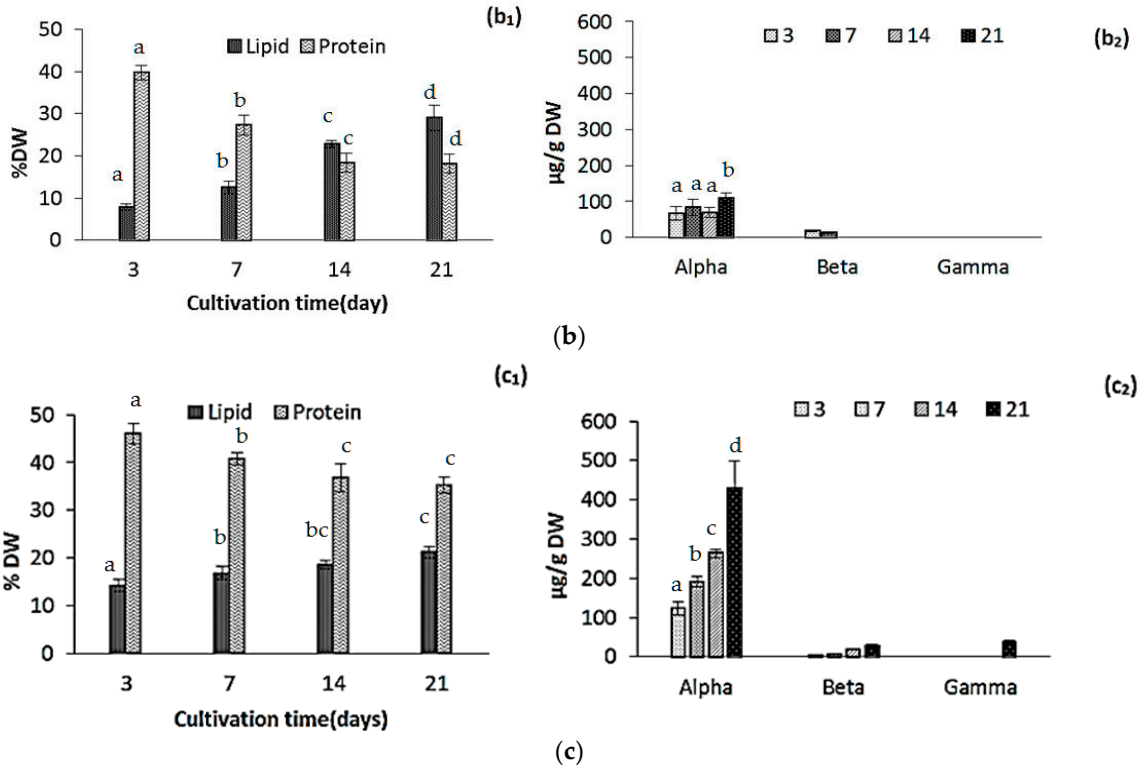


Figure 2. Lipid and protein content ($\mathbf{a_1}$,$\mathbf{b_1}$,$\mathbf{c_1}$) and tocopherol composition ($\mathbf{a_2}$,$\mathbf{b_2}$,$\mathbf{c_2}$) for (**a**) F/2 experiment; (**b**) ICW + F/2 experiment and (**c**) large scale experiment, respectively. The standard errors are presented as bars (n = 2), and different letters indicate significant differences ($p < 0.05$).

Tocopherol composition mostly included α-tocopherol as reported by several studies [34,35]. Total tocopherol content was not significantly different in ICW + F/2 and F/2 experiments during the cultivation (Figure 2a_2,b_2). Amounts of α-tocopherol ranged from 61.2 ± 8.4 µg/g to 113 ± 24 µg/g and 67.3 ± 8.5 µg/g to 109.2 ± 14 µg/g for ICW + F/2 and F/2 cultivated microalgae, respectively. The effects of cultivation time and growth media were not found statistically significant (p = 0.1034). Durmaz et al. [35] showed that decreasing nitrogen concentrations could lead to an increase in α-tocopherol accumulation in *N. oculata*. Concentration of α-tocopherol could be attributed to different factors such as; nitrogen source (e.g., ammonium or nitrate), nitrogen concentration, growth phase and light (L)/dark (D) photoperiod. Microalgae *Nannochloropsis* sp. produce significantly lower quantities of α-tocopherol at 12:12 h (L:D) photo period compared to 24:0 h (L:D) [35]. It could be a reason for lower amounts of α-tocopherol in our experiments compared to the values reported by Durmaz et al. [35].

2.2.2. Fatty Acid Composition

The major fatty acids in *Nannochloropsis* sp. are 14:0, 16:0, 16:1, *n*-7 and EPA, but C18 fatty acids and 20:4 (*n*-6) are present in lower quantities [11–13,15]. Table 3 shows the variation of the fatty acid compositions of *N. salina* grown on two growth media. During the 21 days of cultivation, EPA increased significantly from 4.95% ± 0.78% TFA to 32.0% ± 0.82% TFA and from 7.47% ± 0.23% TFA to 37.1% ± 0.77% TFA, for ICW + F/2 and F/2 cultivated microalgae, respectively. Both cultivation time and growth media had a statistically significant effect on EPA variations at the 95.0% confidence level. Amounts of C 20:4 (*n*-6) were higher in F/2 cultivated microalgae during the course of cultivation. Palmitic acid was the major saturated fatty acid in both experiments. Highest amounts of palmitic acid were detected in samples from the 3rd day of cultivation, which decreased from 48.8% ± 2.59% TFA to 21.3% ± 0.30% TFA and from 43.3% ± 0.98% TFA to 19.1% ± 0.40% TFA after 21 days, for ICW + F/2

and F/2 cultivated microalgae, respectively. Only cultivation time had a statistically significant effect on variation of C16:0 at the 95.0% confidence level. C16:1 (*n*-7) was detected as the main *n*-7 fatty acid in *N. salina* in this study, and it decreased during the cultivation for F/2 cultivated microalgae, but variations were not significant. Effects of cultivation time and growth media on the variations of C18:1 (*n*-7) were not significant at 95.0% confidence level. Total saturates varied significantly from 51.6% ± 3.25% TFA to 25.2% ± 1.63% TFA and from 48.3% ± 2.33% TFA to 25.9% ± 0.62% TFA for ICW + F/2 and F/2 cultivated microalgae, respectively. Variation in total saturated fatty acids was attributed to the cultivation time while both cultivation time and growth media had a statistically significant effect on total *n*-3 PUFA content at the 95.0% confidence level. EPA belongs to a group of fatty acids that are part of the phospholipids, which serve as structural components in the cell wall. Under nutritional limitations, such as nitrogen, cells are unable to resynthesize them or keep the concentration of these components constant [15]. However, with adequate nutrition, cells are capable of synthesizing high amounts of energy rich PUFAs, such as EPA [36], so enrichment of growth media with nitrogen (ammonia) successfully enhanced the EPA contents in *N. salina*.

Table 3. Fatty acid composition of *N. salina* grown on F/2 and ICW + F/2 growth media in laboratory scale. Different letters indicate significant differences ($p < 0.05$).

Cultivation Time (Day)	3		7		14		21	
Fatty Acid	**F/2**	**ICW + F/2**	**F/2**	**ICW + F/2**	**F/2**	**ICW + F/2**	**F/2**	**ICW + F/2**
14:0	1.45 ± 0.44	3.22 ± 0.50	3.52 ± 0.12	0.51 ± 0.05	1.40 ± 0.13	0.56 ± 0.30	2.05 ± 0.50	3.53 ± 0.13
15:0	0.04 ± 0.00	nd	nd	nd	0.54 ± 0.01	1.10 ± 0.40	0.30 ± 0.09	1.46 ± 0.03
16:0	48.8 ± 0.59 ^{a}	43.3 ± 0.98 ^{b}	32.9 ± 0.53 ^{c}	31.9 ± 0.77 ^{c}	23.7 ± 0.40 ^{d}	20.6 ± 0.64 ^{de}	21.3 ± 0.30 ^{de}	19.1 ± 0.40 ^{e}
16:1 (*n*-7)	30.3 ± 0.40 ^{a}	30.9 ± 0.10 ^{a}	24.0 ± 0.09 ^{b}	20.4 ± 0.02 ^{c}	25.1 ± 0.51 ^{b}	23.0 ± 0.02 ^{b}	31.9 ± 0.66 ^{a}	17.7 ± 0.51 ^{c}
16:2 (*n*-4)	0.18 ± 0.01	0.23 ± 0.02	1.24 ± 0.00	0.65 ± 0.00	0.33 ± 0.01	0.76 ± 0.07	0.64 ± 0.02	0.38 ± 0.01
16:3 (*n*-4)	0.11 ± 0.03	3.57 ± 0.09	0.61 ± 0.02	0.56 ± 0.03	0.75 ± 0.04	0.63 ± 0.08	0.24 ± 0.05	1.01 ± 0.04
16:4 (*n*-1)	0.36 ± 0.22	3.35 ± 0.08	0.45 ± 0.04	0.14 ± 0.19	0.44 ± 0.01	0.28 ± 0.10	0.43 ± 0.03	0.66 ± 0.06
18:0	2.35 ± 0.20	1.69 ± 0.19	5.32 ± 0.09	1.82 ± 0.06	2.20 ± 0.01	2.10 ± 0.06	0.51 ± 0.10	1.86 ± 0.07
18:1 (*n*-9)	7.03 ± 0.23	3.31 ± 0.06	9.43 ± 0.08	5.61 ± 0.06	9.06 ± 0.20	6.53 ± 0.06	4.22 ± 0.06	4.38 ± 0.20
18:1 (*n*-7)	0.75 ± 0.20	0.00 ± 0.06	0.45 ± 0.08	6.31 ± 0.60	7.52 ± 0.21	7.50 ± 0.45	0.57 ± 0.06	3.32 ± 0.21
18:2 (*n*-6)	1.13 ± 0.01	0.74 ± 0.06	1.45 ± 0.03	1.35 ± 0.06	2.23 ± 0.08	1.67 ± 0.07	1.63 ± 0.03	1.46 ± 0.09
18:3(*n*-3)	0.64 ± 0.10	0.50 ± 0.01	0.49 ± 0.04	0.46 ± 0.03	0.04 ± 0.02	nd	0.30 ± 0.01	nd
20:1(*n*-7)	0.10 ± 0.00	nd	1.20 ± 0.12	nd	0.10 ± 0.01	nd	0.10 ± 0.01	nd
20:4 (*n*-6)	1.21 ± 0.20	0.73 ± 0.06	1.92 ± 0.05	4.82 ± 0.08	3.82 ± 0.07	5.63 ± 0.08	2.33 ± 0.06	4.91 ± 0.07
20:3 (*n*-3)	0.22 ± 0.08	0.33 ± 0.08	1.57 ± 0.04	0.26 ± 0.02	nd	0.31 ± 0.02	0.17 ± 0.08	0.93 ± 0.02
20:4 (*n*-3)	0.10 ± 0.00	0.33 ± 0.02	0.43 ± 0.03	nd	0.31 ± 0.08	nd	0.10 ± 0.00	nd
20:5 (*n*-3)	4.95 ± 0.78 ^{a}	7.47 ± 0.23 ^{ab}	10.9 ± 0.11 ^{b}	22.2 ± 0.21 ^{c}	19.9 ± 0.42 ^{c}	26.2 ± 0.25 ^{d}	32.0 ± 0.82 ^{e}	37.1 ± 0.77 ^{f}
22:5 (*n*-3)	0.66 ± 0.05	nd	0.40 ± 0.01	0.26 ± 0.04	1.35 ± 0.05	0.14 ± 0.03	0.66 ± 0.03	0.27 ± 0.05
22:6 (*n*-3)	0.46 ± 0.09	0.15 ± 0.04	0.24 ± 0.02	1.04 ± 0.40	nd	0.69 ± 0.40	0.19 ± 0.02	0.16 ± 0.05
∑ SAFA	51.6 ± 3.25 ^{a}	48.3 ± 2.33 ^{a}	41.8 ± 0.63 ^{b}	34.2 ± 1.25 ^{b}	27.9 ± 0.51 ^{c}	24.4 ± 1.44 ^{c}	24.2 ± 1.63 ^{c}	25.9 ± 0.62 ^{c}
∑ *n*-3	6.92 ± 2.41 ^{a}	8.77 ± 0.45 ^{a}	13.6 ± 0.23 ^{b}	24.3 ± 0.70 ^{c}	21.66 ± 0.66 ^{c}	27.4 ± 0.74 ^{d}	33.37 ± 1.0 ^{e}	38.6 ± 1.00 ^{f}

Data are shown as % of fatty acid in total fatty acid composition. Total saturated fatty acids (Σ SAFA), *n*-3 poly unsaturated fatty acid (∑ *n*-3); nd, not detected. Different letters in the same row represent significant difference ($p < 0.05$) for 16:0, 16:1 (*n*-7), 20:5 (*n*-3), total SAFA and *n*-3 fatty acids.

Hu et al. [13] showed an increase of the EPA concentration of *N. salina* in batch cultures supplied with both high and low nitrate concentrations, so effect of environmental factors such as light intensity and temperature should also be considered in development of EPA in this species. It is generally assumed that microalgae have common biosynthetic pathway for production of EPA, that is, desaturation of 18:2 (*n*-6) is conducted by either Δ6 or Δ15 (*n*-3) desaturase trails, resulting in either 18:3 (*n*-6) or 18:3 (*n*-3), which lead to 20:4 (*n*-6) or EPA, respectively [36]. In high-CO_2-grown cells, 18:2 (*n*-6) was of higher relative amount, and available for Δ6 desaturation, which gave rise to an increased production of 18:3 (*n*-6) and subsequently 20:5 (*n*-3) [37]. In the present study, 18:3 (*n*-6) was not detected, suggesting rapid turnover of 18:3 (*n*-6) and higher contents of EPA in the presence of CO_2 as described in previous studies [13,37].

2.2.3. Amino Acid Composition

The amino-acid compositions of *Nannochloropsis salina* grown on two growth media is shown in Figure 3a,b. Nearly all of the amino acids were present in higher concentrations in ICW + F/2 cultivated samples. Concentration of amino acids decreased in the course of cultivation for both experiments. Aspartate, glutamate and arginine were generally found in the highest concentrations. Amino acid profiles are also similar to previous reports on *Nannochloropsis* genus and even microalgae species from different classes [38]. The similarity in the amino acid compositions suggests that the structure of microalgal protein may also be similar. This could indicate that many of the proteins performing specific functions and common to all species (e.g., C-fixation enzymes, membrane proteins associated with light-harvesting pigments) are highly conserved in their amino acid composition throughout the different species [38]. Notable difference was significantly higher amounts of lysine in F/2 cultivated microalgae samples at early stages of cultivation, while amounts of proline and glutamine were higher in ICW + F/2 cultivated samples. Ammonia is generally thought to directly assimilate into the amino acid glutamine [32], which can explain higher concentrations of glutamine in ICW + F/2 cultivated samples (58 mg/g vs. 48 mg/g). In general total amino acid content was higher in ICW + F/2 cultivated samples (Figure 3d), which could be contributed to higher amounts of nitrogen in growth media.

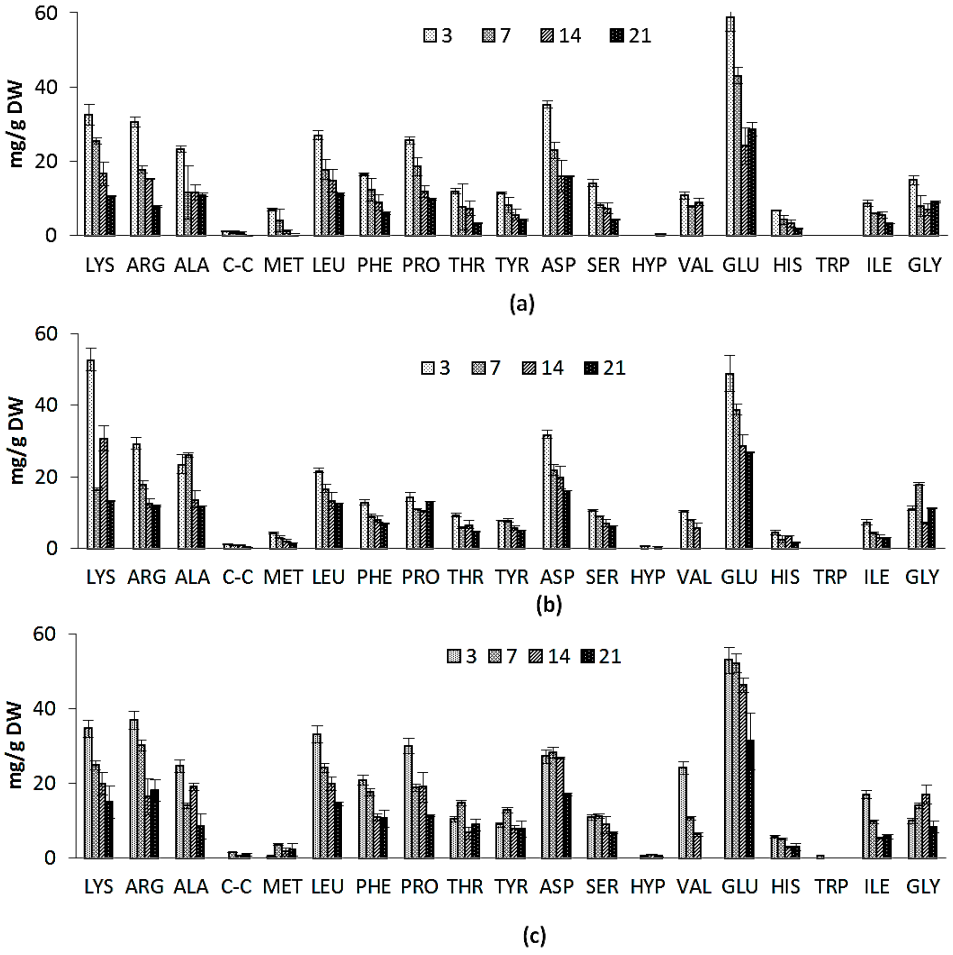
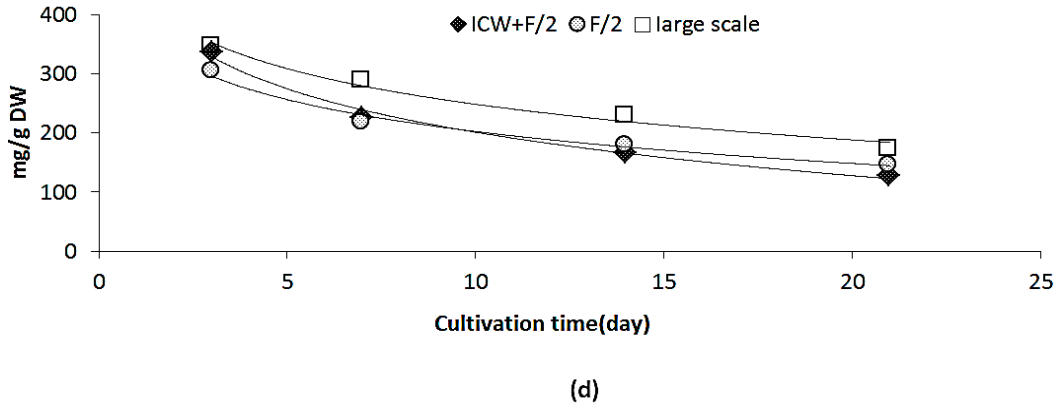


Figure 3. *Cont.*



(d)

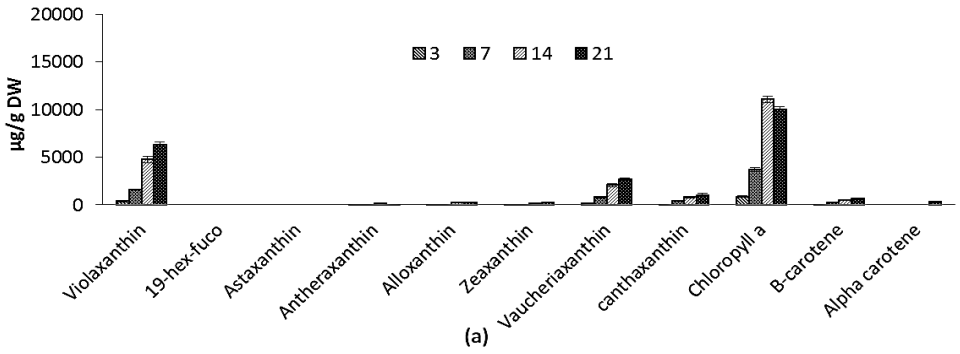
Figure 3. Amino acid composition for: (**a**) ICW + F/2 experiment; (**b**) F/2 experiment; (**c**) large scale experiment and (**d**) total amino acids variations during 21 days of cultivation for ICW + F/2, F/2 and large scale experiments. The standard errors are presented as bars (n = 2).

There was a statistically significant correlation at 95.0% confidence level ($p < 0.05$), between results of protein content and total amino acids in all experiments. The correlation coefficient equals 0.86, indicating a moderately strong relationship between the variables.

2.2.4. Pigments

Pigment composition in *Nannochloropsis* sp. include chlorophyll a (not b or c), and also violaxanthin and vaucheriaxanthin esters, which play a major role in light harvesting as main accessory pigments [34,39]. Other carotenoids include xanthophylls such as canthaxanthin, anteraxanthin, zeaxanthin and carotenes such as β-carotene were also reported in *Nannochloropsis* sp. [39].

Variations in pigment composition of ICW + F/2 and F/2 experiments are shown in Figure 4a,b. Chlorophyll a (Chl) increased during the course of cultivation from 867 ± 84.0 μg/g to 10,030 ± 239 μg/g and from 1048 ± 22.1 μg/g to 8427 ± 419 μg/g for ICW + F/2 and F/2 cultivated microalgae, respectively. Carotenoids include violaxanthin, vaucheriaxanthin esters, alloxanthin, antheraxanthin, cantaxanthin, zeaxanthin and beta carotene in both experiments. During 21 days of experiment, total carotenoids increased from 749 ± 81 μg/g to 11387 ± 874 μg/g and from 797 ± 55 μg/g to 7761 ± 419 μg/g for F/2 and ICW + F/2 cultivated microalgae, respectively. Both cultivation time and growth media significantly influenced the variations of violaxanthin as main carotenoid, while effect of growth media on variations of vaucheriaxanthin was not found significant. The presence of xanthophyll fatty acid esters (mainly vaucheriaxanthin) is characteristic for carotenoid composition in some microalgae such as *Nannochloropsis* spp. [39], and the free-to-esterified vaucheriaxanthin ratio varies in different *Nannochloropsis* spp. from ca. 1:1 to 1:4 [40].



(a)

Figure 4. *Cont.*

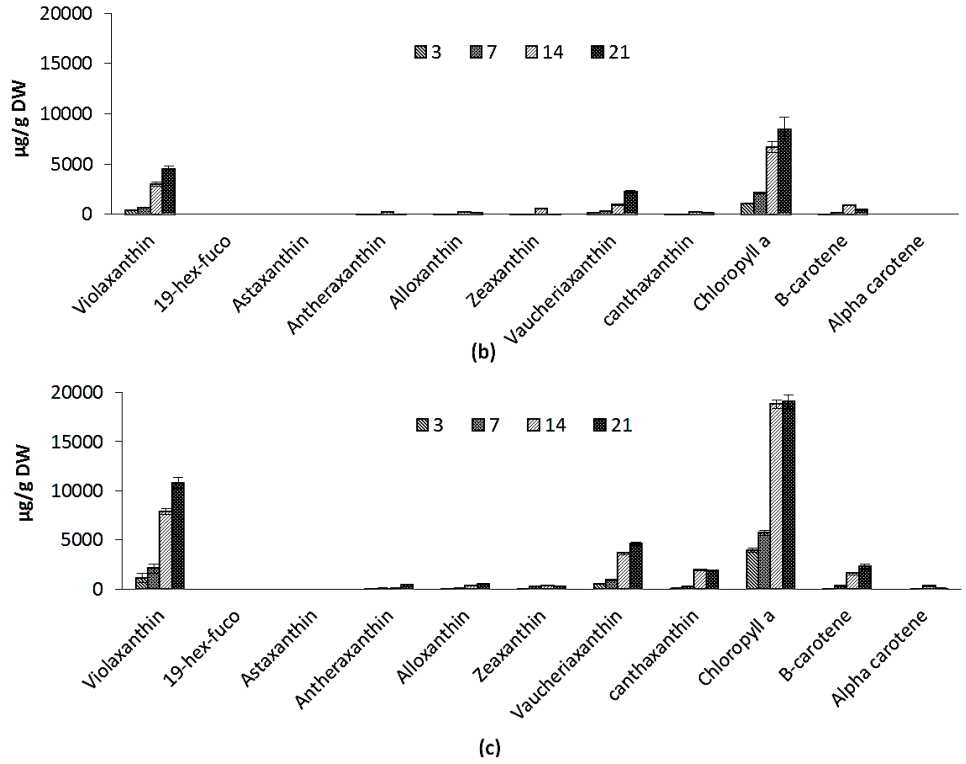


Figure 4. Pigment composition for: (**a**) F/2 experiment; (**b**) ICW + F/2 expeiment; and (**c**) large scale experiment. The standard errors are presented as bars (n = 2).

In this study the ratio of free to esterified vaucheriaxanthin was 1:3.9 for F/2 and 1:3.7 for ICW + F/2 experiments. Like non-esterified vaucheriaxanthin, esterified forms may be attributed to pigment-protein complexes of the thylakoid membranes, but they do not participate in light harvesting in *Nannochloropsis* [39], so vaucheriaxanthin will not follow the variations in light harvesting carotenoids. Car/Chl increased during the cultivation from 0.76 to 0.92 for ICW + F/2 and from 0.86 to 1.13 for F/2 experiments. It was reported previously that considerable rise in Car/Chl along with accumulation of FA takes place similarly and is induced by nitrogen starvation, while there were also reports about accumulation of carotenoids in stressed *Nannochloropsis* cells [39,41]. In this study, nitrogen concentration was higher in ICW + F/2 growth media and resulted in higher chlorophylls and lower carotenoids in the biomass, compared to F/2 growth media. So comparatively higher values of Car/Chl in F/2 cultivated samples can be attributed to the lower levels of nitrogen. It has been clearly demonstrated previously, that in the process of chlorophyll biosynthesis, nitrogen (in form of δ-aminolevulinic acid) is involved as a precursor in the synthesis of pyrol ring in chlorophyll structure [40]. In the presence of a carbon source, concentration of chlorophyll(s) can be directly attributed to the amount of nitrogen (in-organic form) in growth media.

2.3. Changes in Growth and Biomass Composition of Large Scale Cultivated Nannochloropsis salina

2.3.1. Growth Rate and Biomass Productivity

The growth curve for large scale cultivation of *Nannochloropsis salina* using nutrient composition as 20% ICW + 80% F/2 at pH 8.3 ± 0.2 is shown in Figure 1c. Growth started rapidly, but did not reach the stationary phase. Variation in optical density and biomass productivity can be attributed to temperature variations (Figure 1d), which resulted in sedimentation and inhomogeneity of the culture. The average solar radiation values from 2006 to 2010 in Denmark previously reported as 16 KWh·m^{-2} for October–November, which compared to June–July (75 KWh·m^{-2}) is very low [42]. Daily light varied

from 11 h to 9 h during the experiment. Both growth rate and biomass productivity can be affected by low temperature and low daily light hour. For example Sforza et al. [14] reported 30% less growth rate and 28% less biomass concentration for *Nannochloropsis salina* cultivated under light: dark cycle compared to continuous illumination. On the other hand, lower biomass concentration will provide a greater possibility for absorption of light energy in flat panel photo-bioreactor narrow tubes (32 mm). Olofsson et al. [18] reported a relatively constant biomass concentration during two years of cultivation of *Nannochloropsis oculata* in large scale outdoor photo bioreactor, with narrow light path (5 cm). In another study, an increase in the light path length of photo bioreactor from 5 to 10 cm significantly decreased the biomass productivity in *Nannochloropsis gaditana* [21]. Chini Zittelli et al. [17] reported that continuous illumination with artificial light resulted in 37% increase in volumetric productivity of *Nannochloropsis* sp. cultivated in flat panel photo bioreactor. Environmental and operational conditions highly affect the growth and lipid production in *Nannochloropsis* sp. [22] *Nannochloropsis salina* can grow in Denmark's winter time conditions and tolerate the temperature/light variations, but when high biomass productivity (and not chemical composition) is the main target, artificial light will be required. For efficient cultivation of microalgae in a photo bioreactor, optimizing light availability and light intensity is critically required [14,21,22].

2.3.2. Biomass Chemical Composition

Large scale cultivation of *Nannochloropsis salina* influenced the chemical composition of the biomass during the course of cultivation and before reaching the steady state conditions. Cultivation time significantly influenced variations in lipid and protein contents ($p < 0.05$).

Total lipid contents increased from 10.8% $\pm$ 1.2% DW to 21.1% $\pm$ 1.2% DW, but the rate of accumulation was lower comparing to the laboratory scale experiment with the same growth media, which can be explained with lower light and temperature. Olofsson et al. [18] also reported that variations in total lipid content of *Nannochloropsis oculata* can be explained with both light and temperature variations. Highest and lowest lipid productivity was reported in autumn and winter, respectively. Camacho-Rodríguez et al. [21] reported that an increase in the path length width of photo bioreactor from 5 to 10 cm significantly decreased both lipid contents in *Nannochloropsis gaditana* from 28% to 24% DW [21]. The geometry of flat-panel bioreactor is a key factor for lipid productivity along with environmental and operational parameters such as pH and dilution (harvest) rate [21,22].

In our study protein contents decreased from 46.0% $\pm$ 1.3% DW to 33.4% $\pm$ 1.7% DW (Figure 2c$_1$). In general variations in protein contents depends to the growth stage, environmental conditions such as light and temperature and composition of growth media. Protein content is also species-related, which can explain higher protein contents in our results compared to another study which reported the variations of protein contents in *Nannochloropsis occulata* [20]. San Pedro et al. [22] reported that protein content of biomass remained in the range of 49.3% and 30.9% DW for *Nannochloropsis gaditana* cultivated in outdoor pilot-scale raceway ponds. It has also been shown that light path lengths in a photo bioreactor can affect the protein contents in *Nannochloropsis gadiata*, as protein contents increased significantly when light path length increased from 5 to 10 cm [21]. In this study, average protein contents were reported as 45% DW with highest values in the microalgae cultivated at the summer. It can be concluded that several factors such as reactor design and specifications, dilution (harvest) rate, growth media and environmental factors such as temperature and light affect the protein accumulation. On the other hand, the protein accumulation in microalgae is species-specific [3]. In our study, rate of variation in protein content was lower compared to the results of laboratory scale cultivation (54.1% and 27.4%, respectively) as shown in Figure 1d. This could be explained by the fact that large scale cultivation was done during the autumn–winter season when environmental factors such as lower light and temperature and sufficient nitrogen in the growth media [20–22,33] influence the growth and metabolism pathway.

Uptake and utilization of nitrogen are reported to be temperature sensitive [11], so nitrogen level available in the growth media may have to be optimized during the winter season to obtain optimal growth. It can be considered as benefit when protein is the target compound in the biomass.

Large scale cultivation enhanced the concentration of α-tocopherol as it increased to 431 ± 28 μg/g during 21 days of cultivation compared to 109.08 ± 11 in lab scale (Figure 2C_2). This difference could be explained by higher availability of light (even in autumn-winter season) in flat panel photo-bioreactor because of short path length compared to the batch laboratory reactors. On the other hand our values were still low, compared to data reported by Durmaz et al. for *Nannochloropsis oculata*, which was reported to contain 2325.8 ± 39 μg/g α-tocopherol [35]. This lower concentration can be explained by seasonal low photoperiod, which was demonstrated as an important factor, so it could be suggested that in spring-summer season α-tocopherol content will be increased with the same growth media. Durmaz et al. [35] also reported that concentration of tocopherols increased significantly in stationary phase as a response to the nitrogen starvation.

Variations in fatty acid composition resembled the laboratory scale findings for C16:0 and total saturates, which decreased during the cultivation time from 24.9% ± 2.28% TFA to 17.4% ± 0.24% TFA and from 32.0% ± 3.10% TFA to 21.2% ± 0.74% TFA, respectively (Table 4). EPA and total *n*-3 fatty acids increased accordingly, from 23.9% ± 1.78% TFA to 44.2% ± 2.30% TFA and from 27.8% ± 2.30% TFA to 48.1% ± 2.43% TFA, respectively. Opposite of laboratory scale results, variation in C16:1 was not significant. Results of EPA reported in our study are among the highest ever reported for *N. salina* in both laboratory and large scale [11–13,15,18–20,25,32,33]. Several studies reported that low temperature and short day light, which is equal to less solar energy, could enhance the EPA accumulation in *Nannochloropsis* sp. [15,18–22]. The geometry of a photo-bioreactor can also affect the EPA productivity in *Nannochloropsis* sp. [21]. A significant decrease in EPA productivity was obtained when the light path length in the photo-bioreactor increased from 5 to 10 cm, while the highest EPA productivity was obtained in winter [21]. Low temperatures can decrease the cell membrane fluidity, and microalgae counteracted this environmental effect by increased synthesis of polyunsaturated fatty acids such as EPA [11]. It can be concluded that low temperature (12.0 ± 1.5 °C) during large scale cultivation enhanced the accumulation of EPA in *Nannochloropsis salina*, compared to laboratory scale results for the experiment with the same growth media (ICW + F/2). Laboratory scale experiments were carried out at 20 ± 2 °C.

Variations in amino acid composition of large scale cultivated *N. salina* are shown in Figure 3c. To our knowledge not the same study concerning the variations in amino acid composition in the *Nannochloropsis* sp. cultivated in large scale. Glutamate was rated as the main amino acid, while concentration of lysine, arginine, and leucine were nearly the same. It could be concluded that the variations in amino acids in large scale followed the variation in protein contents in laboratory scale experiments. The presented amino acid composition for large scale cultivated *Nannochloropsis salina* was an ideal composition for aquaculture feed formulas [38]. Total amino acid content also decreased during the cultivation with a gentler slope than ICW + F/2 laboratory scale experiment (Figure 3d).

Pigment composition of large scale cultivated *Nannochloropsis salina* resembled the results, which were discussed previously for laboratory scale cultivated species. Variations in pigment composition are shown in Figure 4c. Chlorophyll content increased during the course of cultivation from 3944 ± 200 μg/g to 19,020 ± 720 μg/g. During 21 days of experiment, total carotenoids increased from 2038 ± 48 μg/g to 20,797 ± 1048 μg/g. Amounts of beta-carotene increased significantly from 26.9 ± 48 μg/g to 2284 ± 234 μg/g. Other carotenoids including vaucheriaxanthin, canthaxanthin, and zeaxanthin also increased during the cultivation time. Free to esterified vaucheriaxanthin ratio was 1:4.2. Car/Chl increased during the cultivation from 0.5 to 1.1. This finding can be explained by environmental stresses such as low temperature and photoperiod, which may influence the light harvesting pigments, i.e., mainly violaxanthin in *Nannochloropsis* sp. [15,36].

Table 4. Fatty acid composition of *N. salina* cultivated in flat-panel photo-bioreactor. Different letters indicate significant differences ($p < 0.05$).

Cultivation Time (Day)	3	7	14	21
Fatty Acid				
14:0	5.28 ± 0.57	4.20 ± 0.50	3.58 ± 0.37	3.37 ± 0.37
15:0	0.67 ± 0.44	0.38 ± 0.04	0.26 ± 0.04	0.24 ± 0.04
16:0	24.9 ± 2.28 [a]	19.9 ± 0.59 [b]	18.5 ± 0.19 [b]	17.4 ± 0.24 [b]
16:1 (*n*-7)	26.5 ± 2.59 [a]	28.7 ± 0.30 [a]	28.1 ± 0.44 [a]	26.4 ± 0.19 [a]
16:2 (*n*-4)	0.18 ± 0.03	0.23 ± 0.02	0.10 ± 0.02	0.10 ± 0.00
16:3 (*n*-4)	0.20 ± 0.01	0.45 ± 0.02	0.22 ± 0.03	0.10 ± 0.02
16:4 (*n*-1)	0.44 ± 0.03	1.01 ± 0.02	0.50 ± 0.02	0.21 ± 0.02
18:0	1.91 ± 0.22	0.37 ± 0.08	0.26 ± 0.03	0.47 ± 0.02
18:1 (*n*-9)	6.06 ± 0.76	3.93 ± 0.19	1.87 ± 0.10	1.76 ± 0.21
18:1 (*n*-11)	2.62 ± 0.20	1.31 ± 0.06	0.94 ± 0.03	0.24 ± 0.00
18:2 (*n*-6)	2.39 ± 0.23	1.30 ± 0.06	0.16 ± 0.01	0.88 ± 0.03
18:3 (*n*-3)	0.10 ± 0.01	nd	nd	nd
20:1 (*n*-9)	0.10 ± 0.02	0.11 ± 0.01	nd	nd
20:1(*n*-7)	0.24 ± 0.02	nd	0.14 ± 0.01	nd
20:4 (*n*-6)	0.05 ± 0.02	nd	nd	0.13 ± 0.01
20:3 (*n*-3)	1.10 ± 0.03	6.45 ± 0.06	3.38 ± 0.05	2.75 ± 0.03
20:4 (*n*-3)	0.61 ± 0.01	0.10 ± 0.08	1.02 ± 0.34	0.91 ± 0.01
20:5 (*n*-3)	23.9 ± 1.78 [a]	30.1 ± 1.21 [b]	39.3 ± 1.10 [c]	44.2 ± 2.30 [d]
22:1 (*n*-11)	nd	0.16 ± 0.03	0.10 ± 0.03	0.12 ± 0.03
21:5 (*n*-3)	1.82 ± 0.39	0.81 ± 0.35	1.03 ± 0.01	0.09 ± 0.01
22:5 (*n*-3)	0.32 ± 0.05	0.23 ± 0.03	0.07 ± 0.01	0.06 ± 0.02
22:6 (*n*-3)	nd	0.12 ± 0.20	0.10 ± 0.04	0.10 ± 0.01
$\sum$ SAFA	32.0 ± 3.10 [a]	24.5 ± 1.21 [b]	22.3 ± 0.72 [b]	21.2 ± 0.74 [b]
$\sum$ *n*-3	27.8 ± 2.30 [a]	37.7 ± 2.00 [b]	44.9 ± 1.52 [bc]	48.1 ± 2.43 [c]

Data are shown as % of fatty acid in total fatty acid composition. Total saturated fatty acids (Σ SAFA), *n*-3 poly unsaturated fatty acid ($\sum$ *n*-3); nd, not detected. Different letters in the same row represent significant difference ($p < 0.05$) for 16:0, 16:1 (*n*-7), 20:5 (*n*-3), total SAFA and *n*-3 fatty acids.

3. Materials and Methods

3.1. Chemicals and Reagents

Standards of fatty acids, amino acids and tocopherols were purchased from Sigma (St. Louis, MO, USA) and Fluka (Deisenhofen, Germany). Standards of pigments were purchased from DHI (Hørsholm, Denmark). HPLC grade acetonitrile, heptane, isopropanol, methanol and acetone were purchased from Sigma and Fluka. HPLC grade water was prepared at DTU Food using Milli-Q® Advantage A10 water deionizing system from Millipore Corporation (Billerica, MA, USA).

3.2. Growth Media

Industrial process water (ICW) was collected from effluent stream of an anaerobic methanogenic conversion reactor (Novozymes plant, Kalundborg, Denmark). Batches of industrial process water was filtered using an out-side-in dynamic cross flow microfiltration BioBooster system from Grundfos A/S (Bjerringbro, Denmark) equipped with 0.2 µm ceramic disc filters and stored at −20 °C prior to use. Chemical composition of the industrial process water is shown in Table 1. The basic culture medium was a conventional algae F/2 growth media powder based on Guillard & Ryther [24], which was purchased from Varicon Aqua solutions (Worcs, UK). Salt brine was prepared from refined salt (Scan salt A/S, Kolding, Denmark) and UV-sterilized and micro-filtrated tap water, which was added to the growth media in order to achieve a final salinity of 25 g/L.

3.3. Laboratory Scale Cultivation of Microalgae

Microalgae strain *Nannochloropsis salina* (Strain Number: 40.85) was obtained from culture collection of algae (SAG), University of Gottingen. The strain was cultivated in 1–5 L Schott bottles, while shaking gently in an orbital platform shaker (Heidolf instruments GmbH, Schwabach, Germany). During the cultivation, all reactors were continuously aerated with 2% carbon dioxide/air mixture under fluorescent lights with an intensity of 100 µmol photon $m^{-2} \cdot s^{-1}$ and 12:12 (day:night) photoperiod. Light intensity was measured using a Li-190 quantum sensor (LI-COR, Inc., Lincoln, NE, USA). Online monitoring and control of pH was performed by Milwaukee MC-122-pH controller (Milwaukee Electronics, Szeged, Hungary) equipped with solenoid valve to control the stream of CO_2. Control of temperature was done by an aluminum plate connected to cold water circulation system and the temperature was kept constant at 23 $\pm$ 2 °C. Preliminary experiments were performed to explore growth rates at different levels of substitution of F/2 stand growth media with ICW (20%, 30%, and 40%), and pH (6.3 $\pm$ 0.2, 7.3 $\pm$ 0.2, and 8.3 $\pm$ 0.2). Table 2 shows the type and amounts of nitrogen in each growth media. Cultivation was repeated for *N. salina* on 20% ICW + 80% F/2 and standard F/2 media in 10 L Schott bottles at pH 8.3 $\pm$ 0.2 and with the same conditions as mentioned before. During the cultivation and at each sampling point, 0.25 L of culture was taken and samples were centrifuged at 10,000 g. Then resulting biomass pellet was washed twice with deionized water. Resulting biomasses were freeze dried immediately to moisture content less than 1% DW. Measurement of moisture was done by an AD 4714A moisture analyzer (A&D Company, Tokyo, Japan). Samples stored at −20 °C prior to chemical analysis.

N. salina cultivated on 20% ICW + 80% F/2 was transferred to Kalundborg microalgae facility (Kalundborg, Denmark) for large scale experiment.

3.4. Large-Scale Cultivation of Microalgae

Large scale cultivation was done at the Kalundborg microalgae facility using flat panel photo-bioreactor system, type hanging garden from Ecoduna produktions GmbH (Bruck/Leitha, Austria). The photo-bioreactors were designed to track the sun thereby allowing the algae to grow most efficiently on natural sunlight. No artificial light was used during the large scale experiment. The system base unit includes 12 hollow chamber sheets (width 2100 mm; height 5600 mm and depth 32 mm) which are assembled on guide rails. The microalgae suspension was transferred into the sheets and was continuously circulated through the module. Each sheet was sealed off with a plastic cover on the top and with a deflecting profile on the bottom and supplied with nutrient stream from the bottom deflecting profile on the lateral input. The nutrient stream was pumped through the sheets by means of hydrostatic pressure and the gas lift effect, as a result, no additional energy is required. In these specific reactors, surface hit by sunlight is multiplied and the light is being distributed so the irradiation will never be too high. All of the sheets of a module were interconnected via a hose system thus ensuring the obstruction-free transfer of the nutrient solution. Details of the flat panel bioreactor are shown in Figure 5. Air stream was filtered through 0.2 µm membrane filter and mixed with pure CO_2 from Cylinder (purity 99.5% v/v). A mixture of air and 2% v/v CO_2 was then introduced from below to ensure gentle but effective mixing of nutrients and algae to control the pH which was adjusted on 8.5 $\pm$ 0.5. This Gas stream drives a gentle liquid flow (1 $L \cdot min^{-1}$ via an "air-lift effect" so the system does not require pump unit. Cultivation was done in batch mode and two separate units were used. At each sampling point, 2 L of culture was taken and samples were centrifuged at 10,000 g. Biomass samples were washed twice with distilled water and then immediately freeze dried and stored at −20 °C prior to the analysis. Cultivation was done during the period of October–November 2015. The culture was kept at steady state basis, by continuous biweekly harvest and feeding of new growth media after day 14.

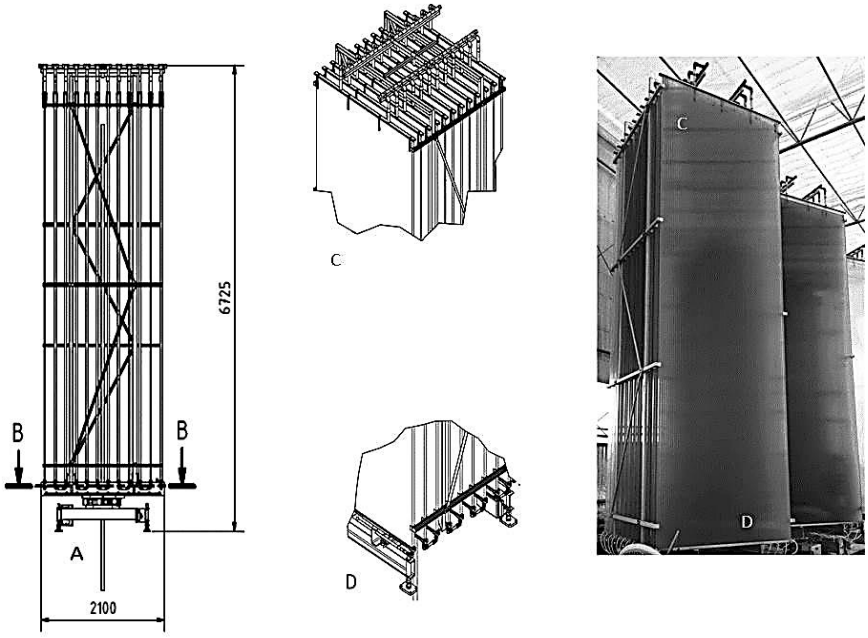


Figure 5. Schematic of flat panel photo-bioreactor which was used for the large scale cultivation experiment. (**A**) Rotating system; (**B**) ground level; (**C**) top part; (**D**) bottom part. Every unit includes 12 sheets. Sheet dimensions: width 2100 mm; height 5600 mm and depth 32 mm.

3.5. Analytical Methods

3.5.1. Growth Rate

Growth rate was monitored by daily detection of optical density at 750 nm.

3.5.2. Protein and Amino Acids

The protein content in the microalgae samples was estimated using a modified Micro biuret method [43] with some modification. One mL of 0.5 M NaOH aqueous solution was added to 10 mg of sample and extraction was carried out at 80 °C for 10 min. Samples were centrifuged at 5000 g and supernatants were moved to new tubes. Extraction was repeated 3 times and all extracts were mixed together before analysis. For protein content estimation, 200 µL of copper sulphate (0.21% $CuSO_4 \cdot 5H_2O$ in 30% NaOH aqueous solution) was mixed with 300 µL of the extract and 500 µL of water. Color formation was monitored at 310 nm. Bovine serum albumin (BSA) was used as the standard for calibration curves.

The amino acid composition was analyzed using EZ:faast™ amino acid analysis kit (Phenomenex Inc., Torrance, CA, USA). For protein hydrolysis around 30 mg of microalgae was weighted in microwave glass vials. The samples were first overlaid with nitrogen and then hydrolyzed in 6 M HCl in microwave oven (Anton Paar GmbH, Graz, Austria) for 60 min at 110 °C. Following individual cleanup-step for removing matrix interference, amino acids derivatization was done. The amino acid composition was determined by liquid chromatography with a Agilent 1100 series LC/MSD Trap mass spectrometry (Agilent technologies, Hørsholm, Denmark) using EZ:faast™ LC-MS column (250 × 3.0 mm, Phenomenex, Torrance, CA, USA).

3.5.3. Lipids, Fatty Acid and Tocopherols

Lipids were extracted in chloroform, methanol and water, as described by Bligh and Dyer using 200 mg of dried microalgae biomass [44].

Fatty acid profile was analyzed according to the method (FAME) based on the AOCS official method [45]. Around 1 g of Bligh and Dyer extract was weighted in methylation glass tube and was evaporated to dryness under a gentle stream of nitrogen. 100 µL of internal standard solution (2% *w*/*v* C23:0 in heptane), 200 µL of heptane with BHT (0.01% *w*/*v*), 100 µL of toluene and 1 mL of borontriflouride in methanol (BF_3-MeOH) was added. Samples were mixed and methylated in microwave oven (Microwave 3000 SOLV, Anton Paar, Ashland, VA, USA) for 10 min at 100 °C and power of 500 watts and then cooled down for 5 min. 1 mL of saturated salt water (NaCl) and 0.7 mL of heptane with BHT were added. After the separation of heptane the upper phase of the sample (around 0.7 mL) was transferred into vials. Samples were analyzed by gas chromatography system (HP-5890 A, Agilent Technologies, Santa Clara, CA, USA). Fatty acid methyl esters were separated and detected by the GC column Agilent DB wax 127-7012 (10 µm × 100 µm × 0.1 µm), from Agilent technologies (Santa Clara, CA, USA).

Analysis of tocopherols and toco-trienols was done by using LC-FLD. 3 g of Bligh and Dyer extract were weighted and evaporated to dryness under a gentle stream of nitrogen. Dry sample was mixed with 1 mL of heptane and then transferred to HPLC vials. Analysis was done based on the AOCS official method [45] using Agilent 1100 Liquid Chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a fluorescence detector, with the excitation wavelength set at 290 nm and emission wavelength at 330. The separation was carried out by a Spherisorb column 150 mm × 46 mm × 3 µm particle size (Waters Corporation, Milford, MA, USA), using mixture of isopropanol and heptane (0.5:99.5) as mobile phase.

3.5.4. Pigments

Extraction and analysis of the pigments was done using the method described by Safafar et al. [34]. Samples were extracted by methanol containing BHT (butylated hydroxyl toluene) in sonication bath (Branson ultrasonics, Danbury, CA, USA) at a temperature lower than 5 °C for 15 min. Samples were analyzed by HPLC using Agilent 1100 Liquid Chromatograph equipped with a DAD. The separation was carried out in a Zorbax Eclipse C8 column 150 mm × 46 mm × 3.5 µm from Phenomenex. The mobile phase was a mixture of solvent A (70% methanol + 30% of 0.028 M tertiary butyl ammonium acetate in water) and solvent B (methanol) at a flow rate of 1.1 mL·min^{-1}. Total acquisition time was 40 min. Identification of peaks and calibration was done by individual standard for each pigment. Detection for carotenoids, chlorophylls and internal standard (BHT) was done at 440 nm, 660 nm, and 280 nm, respectively.

3.5.5. Statistical Analysis

All experiments were repeated two times independently, and data were recorded as the mean. Results were evaluated using ANOVA to test the effect of time and growth media for lab scale and time for large scale experiments. Multiple comparison procedure based on Fisher's least significant difference (LSD) was used to discriminate among the means at the 95.0% confidence level. All statistical analyses were done by STATGRAPHICS software, version Centurion XVI (Stat point Technologies Inc., Warrenton, VA, USA).

4. Conclusions

Cultivation of *N. salina* in a mixture of industrial process water and F/2 standard growth media enhanced the EPA content compared to F/2 growth media. Large-scale cultivation of *Nannochloropsis salina* in a flat panel photo-bioreactor confirmed the laboratory-scale findings. The algae growth rate at winter condition of Denmark was slow, but results revealed that large-scale cultivation of *Nannochloropsis salina* in these conditions could improve the nutritional properties such as EPA, protein, α-tocopherol and carotenoids compared to culturing in lab scale. *Nannochloropsis salina* is a good candidate for large-scale cultivation in the autumn–winter climate in Denmark. Resulting biomass from *Nannochloropsis salina* could be a good source of EPA, amino acid, tocopherols and carotenoids for

the aquaculture feed industry. The biomass could also be a rich source of these bioactive compounds for the food industry, but this may require further extraction and fractionation of the biomass.

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Abbreviations

The following abbreviations are used in this manuscript:

EPA	Eicosa pentanoic acid
ICW	Industrial process water
BHT	Butylated hydroxyl toluene
FPPBR	Flat panel photo bioreactor

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Paper 4

Enhancement of protein and pigment content in two *Chlorella* sp. cultivated on industrial process water

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Enhancement of Protein and Pigment Content in Two *Chlorella* Species Cultivated on Industrial Process Water

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Abstract: *Chlorella pyrenoidosa* and *Chlorella vulgaris* were cultivated in pre-gasified industrial process water with high concentration of ammonia representing effluent from a local biogas plant. The study aimed to investigate the effects of growth media and cultivation duration on the nutritional composition of biomass. Variations in proteins, lipid, fatty acid composition, amino acids, tocopherols, and pigments were studied. Both species grew well in industrial process water. The contents of proteins were affected significantly by the growth media and cultivation duration. Microalga *Chlorella pyrenoidosa* produced the highest concentrations of protein (65.2% ± 1.30% DW) while *Chlorella vulgaris* accumulated extremely high concentrations of lutein and chlorophylls (7.14 ± 0.66 mg/g DW and 32.4 ± 1.77 mg/g DW, respectively). Cultivation of *Chlorella* species in industrial process water is an environmentally friendly, sustainable bioremediation method with added value biomass production and resource valorization, since the resulting biomass also presented a good source of proteins, amino acids, and carotenoids for potential use in aquaculture feed industry.

Keywords: industrial process water; microalgae biomass; lipids; lutein; chlorophyll; *Chlorella*; amino acids; tocopherols; fatty acids

1. Introduction

Algae are a diverse group of autotrophic organisms that have created immense interest due to their specific growth requirements such as efficient use of light energy, their ability to grow rapidly, fix atmospheric CO_2, and produce more biomass per acre than land plants [1]. Microalgae are known as a potentially sustainable source of feedstock for fuel, food, chemicals, feed and even for the pharmaceutical industry [2,3].

Chemical composition of algal biomass is well studied and contains proteins, carbohydrates, lipid, pigments, vitamins, antioxidants, and trace elements. Proteins can be the most dominant nutritious compounds in some cyanobacteria such as *Arthrospira* sp., and microalgae such as *Chlorella* sp. [3,4]. Broadly microalgae present all essential amino acids such as arginine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, and valine [4]. In general, the amino acid composition in most microalgae is more or less the same as terrestrial plants and animal food products. High protein content, as well as proper amino acid composition, makes microalgal biomass a promising alternative ingredient in the formulation of aquaculture feed

[5,6]. Moreover, there has been interest from both a research and economic point of view for microalgae such as *Chlorella* sp. as an alternative source of amino acids and proteins even for human nutrition [7].

Microalgae also contain other valuable bioactive compounds such as the accessory pigments carotenoids. The main carotenoids in microalgae include β-carotene, lutein, astaxanthin, fucoxanthin, violaxanthin and zeaxanthin. Astaxanthin and lutein are used for enhancement of the pigmentation in fish and as a colorant for foods, drugs, and cosmetics, respectively [5,8]. It is also known that *Chlorella* sp. is rich in lutein [3], with an accumulation of lutein of up to 0.45% of dry cell weight in some species, which then makes lutein the primary carotenoid in these green algae [9].

Cultivation of microalgae in wastewater provides an efficient means of nitrogen and phosphorus recycling for the production of lipids and proteins and also bioremediation of effluents before their discharge in the environment [10–12]. The wastewater should be free of pollutants such as heavy metals, which would be accumulated in the algal biomass, and finally in the animal tissues, if the algal biomass was targeted as a food or feed ingredient.

Anaerobic digestion represents promising, cost-effective wastewater treatment technology with widespread applications [13]. Under anaerobic conditions, methanogenic conversion of organic compounds to biogas will result in methane, CO_2, and an effluent which contains inorganic compounds such as ammonia and phosphorous. The conversion process happens in an anaerobic sludge tower reactor with internal circulation (ICT), so the effluent is called IC water (ICW). The growth performance of microalgae cultivated in ICW depends on several factors including the characteristics of algal species (e.g., ammonia tolerance) and properties of the anaerobic digestion effluent [13,14]. An efficient phycoremediation of primarily treated domestic effluents by *Chlorella* spp. was reported previously in several studies [15–17]. These reports suggest phycoremediation as a feasible strategy to reduce the release of organic and inorganic compounds into natural waters, and valorize the resulting biomass by converting the waste water nutrients into bioactive compounds such as protein, lipids, and pigments.

Chlorella is one of the most promising microalgal genuses from both a scientific and a commercial point of view. *Chlorella vulgaris* was first described by Beijerinck in 1890 [18]. After that, a large number of *Chlorella* species were isolated and characterised [9]. *Chlorella vulgaris* and *Chlorella pyrenoidosa* were reported as high protein containing species among other microalgae belonging to Chlorophytes [4].

This study aims to investigate the effects of different concentrations of ICW, and cultivation duration (time) on nutritional composition (regarding protein, amino acids, and carotenoids) of final biomass. Furthermore, this study evaluated which growth condition and harvest time would be optimal for highest protein productivity, since the target was the production of algal biomass applicable as an ingredient for aquaculture feed; rich in proteins and carotenoids and with moderate lipid contents. The optimal growth condition was determined by laboratory batch experiments to provide necessary reference data to be scaled up to industrial scale.

2. Materials and Methods

2.1. Chemicals and Reagents

Standards of fatty acids, amino acids and tocopherols were purchased from Sigma (St. Louise, IL, USA) and Fluka (Deisenhofen, Germany). Standards of pigments were obtained from DHI (Hørsholm, Denmark). HPLC grade acetonitrile, heptane, isopropanol, methanol, and acetone were purchased from Sigma and Fluka. HPLC grade water was prepared at DTU Food using Milli-Q® Advantage A10 water deionizing system from Millipore Corporation (Billerica, MA, USA).

2.2. Growth Media

Industrial process water (ICW) was collected from the effluent stream of an anaerobic methanogenic conversion reactor (Novozyme's plant, Kalundborg, Denmark). Batches of industrial process water were filtered using an out-side-in dynamic cross-flow microfiltration Bio Booster

system from Grundfos A/S (Bjerringbro, Denmark) equipped with 0.2 μm ceramic disc filters and stored at −20 °C before use. The chemical composition of the industrial process water is shown in Table 1a. Table 1b shows the concentrations of nitrogen and phosphorus at each medium tested. Deionized sterile water was used for the microalgal growth in the laboratory.

Table 1. (**a**) Chemical composition of industrial process water; and (**b**) type and amounts of nitrogen and phosphorus in each growth medium.

(a)

Item	Unit	Amount
pH	-	8.1
Suspended solids	mg/L	20
Total N	mg/L	190
Ammonia + ammonium-N	mg/L	150
Nitrite + nitrate	mg/L	<0.1
Total P	mg/L	11
Sulphate	mg/L	3.6
Total cyanide	μg/L	2.5
Total Alkalinity	mmol/L	62.5
EDTA (Ethylenediaminetetraacetic acid)	mg/L	<0.5
Sodium(Na)	mg/L	1500
Cadmium (Cd)	μg/L	<0.05
Copper (Cu)	μg/L	3.4
Iron (Fe)	mg/l	0.23
Cobolt (Co)	μg/L	<0.5

(b)

Growth media*	NH_4^+-N	Total P
34 % ICW	50	3.5
67 % ICW	100	6.8
100 % ICW	150	11

All values are in mg/L*

2.3. Microalgal Growth Experiments

A strain of *Chlorella pyrenoidosa* (ATCC®75668™) was provided by American Type Culture Collection (ATCC) in partnership with LGC Standards (LGC standards-ATCC), Middlesex, UK. *Chlorella vulgaris* (SAG 211-81) was provided by Culture Collection of Algae at Göttingen University (SAG), Göttingen, Germany.

Both strains were cultivated in 1-5 L Schott bottles. All reactors were continuously aerated with 2% carbon dioxide/air mixture under fluorescent lamp illumination (Green-line A/S, Maribo, Denmark) with an irradiance of 200 μmol photon $m^{-2} \cdot s^{-1}$ and 14:10 (h) photoperiod. Light intensity was measured using a Li-190 quantum sensor (LI-COR, Inc., Lincoln, NE, USA). Online monitoring and control of pH were performed by Milwaukee MC-122-pH controller (Milwaukee Electronics, Szeged, Hungary) equipped with a solenoid valve to control CO_2 addition. Temperature was controlled by an aluminum plate connected to cold water circulation system and the temperature

was kept constant at 23 ± 2 °C. Preliminary experiments were performed to explore growth at different concentrations of ICW (34%, 67% and 100%) diluted with de-ionized water, at constant pH 7.3 ± 0.2. During the cultivation and at each sampling point, 0.25 L of culture was taken and samples were centrifuged at 10,000× *g*. Resulting biomass was freeze-dried and stored at −20 °C prior to chemical analysis.

2.4. Analytical Methods

2.4.1. Growth Curve

Microalgal growth was monitored by daily detection of optical density at 750 nm, which is outside the range of absorbance by the pigments as suggested by Griffiths et al. [19]. For the preparation of laboratory samples at each sampling point, 0.25 L of culture was taken after homogenization by shaking for 5 min and centrifuged at 10,000× *g*. The resulting biomass pellet was washed twice with deionized water. The resulting pellet was washed twice with deionized water and freeze dried immediately until a moisture content below 1% DW was reached. Measurement of moisture was done by an AD 4714A moisture analyser (A&D Company, Tokyo, Japan). Samples were stored at −20 °C before chemical analysis. Dry matter (DM) was calculated using the following equation obtained from a standard curve of concordance between dry matter and optical density at 750 nm (OD_{750}):

$$DM\ (\mathrm{g \cdot L^{-1}}) = 0.376\ OD_{750} + 0.005 \quad (1)$$

2.4.2. Proteins and Amino Acids

The protein content in the microalgal samples was estimated using a modified Micro biuret method described by Safafar et al. [14].

The amino acid composition was analysed using EZ:fast™ Amino acid analysis kit (Phenomenex Inc., Torrance CA, USA). Separation was done by liquid chromatography using Agilent 1100 series LC/MSD Trap mass spectrometry (Agilent Technologies, Hørsholm, Denmark) with a EZ:fast™ Liquid chromatography-Mass spectroscopy (LC-MS) column (250 × 3.0 mm, Phenomenex), as described previously by [14].

2.4.3. Lipids, Fatty Acid, and Tocopherols

Lipids were extracted with chloroform:methanol solvent mixture for two hours, as described in Bligh and Dyer [20], using 200 mg dry biomass.

Fatty acid profile was analyzed according to the American Oil Chemists' Society (AOCS) official method; Ce 1i-07 [21]. Around 1g of extract was weighed in methylation glass tube and was evaporated to dryness under a gentle stream of nitrogen. Then, 100 µL of internal standard solution (2% *w*/*v* C23:0 in heptane), 200 µL of heptane including 0.01% *w*/*v* butylated hydoxy toluene (BHT) as antioxidant, 100 µL of toluene and 1 mL of boron trifluoride in methanol (BF_3-MeOH) was added. Samples were mixed and methylated in the microwave oven (Microwave 3000 SOLV, Anton Paar) for 10 min at 100 °C and power of 500 W and then cooled down for 5 min. Then, 1 mL of saturated salt water (NaCl) and 0.7 mL of heptane with BHT were added. After the separation of heptane, the upper phase of the sample (around 0.7 mL) was transferred into vials. Samples were analyzed by gas chromatography system (HP-5890 A, Agilent Technologies, Santa Clara, CA, USA). Fatty acid methyl esters were separated and detected by the GC column Agilent DB-wax (10 m × 100 µm × 0.1 µm), from Agilent Technologies (CA, USA). Fatty acids are reported as % of total fatty acids.

Analysis of tocopherols and tocotrienols was done by Liquid Chromatograph equipped with Fluorescence Detector(LC-FLD). Approximately 3 g of Bligh and Dyer extract was weighted and evaporated to dryness under a gentle stream of nitrogen. Dry sample was mixed with one mL of heptane and then transferred to HPLC vials. The analysis was performed based on the AOCS official method; Ce 8-89 [21], using Agilent 1100 Liquid Chromatograph (Agilent Technologies, CA,

USA), equipped with a fluorescence detector, with the excitation wavelength of 290 nm and emission wavelength of 330. The separation was carried out by a Spherisorb column 150 mm × 46 mm × 3μm particle size (Waters Corporation, Massachusetts, USA), using a mixture of isopropanol and heptane (0.5:99.5) as the mobile phase. Quantification was done based on external calibration and by series of five different standard concentrations of α-, γ-, and δ-tocopherol.

2.4.4. Pigments

Extraction and analysis of the pigments were done by the method described by Safafar et al.[22] Samples were extracted by methanol containing BHT in a sonication bath (Branson Ultrasonics, Danbury, CA, USA) at 5 ± 1 °C for 15 min. Analyses were performed by an Agilent 1100 Liquid Chromatograph equipped with a Diode Array Detector (DAD). Separation was carried out on a Zorbax Eclipse C8 column 150 mm × 46 mm × 3.5 μm from Phenomenex. Identification was done by using DHI pigment standard mix. Quantification was done based on external calibration and by series of five different standard concentrations for each individual pigment.

2.4.5. Statistical Analysis

Cultivation experiments were repeated two times in one and five litre flasks, respectively. All analytical procedures were performed on the samples from 5 L flasks and repeated two times independently. The data are recorded as the mean. Results were evaluated using ANOVA to test the effect of time and growth media. Bonferroni multiple comparison procedure was used to discriminate among the means at the 95.0% confidence level. All statistical analyses were done by STATGRAPHICS software, version Centurion XVI (Stat point Technologies, Inc., Warrenton, VA, USA).

3. Results and Discussion

3.1. Growth and Biomass Production

Both species grew on industrial process water. Biomass increment and growth trends for *C. vulgaris* and *C. pyrenoidosa* are shown in Figure 1a,b, respectively. Biomass increased over the cultivation time in all experiments until the end, day 16. The biomass was significantly higher for *C. vulgaris* cultivated in 67% ICW, compared to 34% and 100% ICW experiments. (Figure 1a). For the *C. pyrenoidosa* experiment, on the other hand, higher biomass was measured in 100% ICW, compared to 34% and 67% experiments (Figure 1b). The differences in the optimal concentration of ICW between the two species can be attributed to the maximum tolerated level of NH_4^+-N for these two *Chlorella* species, so that *C. vulgaris* tolerates lower concentrations of ammonia in growth medium. The ammonium concentration is known to be a critical factor for growth and biomass productivity of *Chlorella* sp. [23]. He et al. [10] reported a positive correlation between *C. vulgaris* cell density and concentration of ammonia at levels of 17 to 143 mg·L^{-1} NH_4^+-N., while higher levels of ammonia dropped the growth rate. Cho et al. [24], reported high biomass productivity (0.4 g·L^{-1}·d^{-1}) and biomass production = 3 g·L^{-1}) for *Chlorella* sp., cultivated on tenfold diluted wastewater from an anaerobic digestion in which the concentration of NH_4^+-N was 220 mg·L^{-1}.

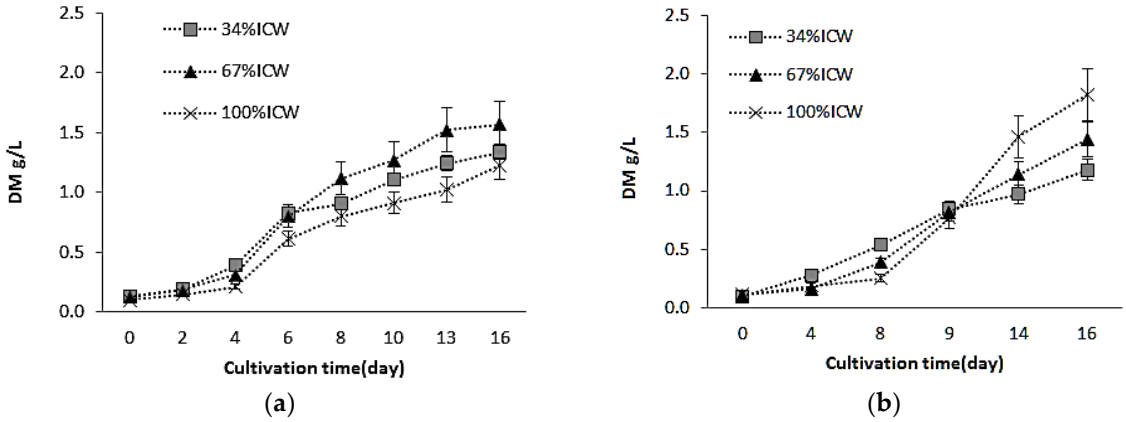


Figure 1. Growth curve during 16 days of cultivation in internal circulation water (ICW): (**a**) *Chlorella vulgaris*; (**b**) *Chlorella pyrenoidosa*. The error bars represent the standard deviations.

In our study, concentrations of NH_4^+-N ranged from 50 to 150 mg·L^{-1}, which are in the optimum range reported for *C. vulgaris*. Moreover, other studies [2] suggested a substrate inhibition at high nitrate concentrations. It was reported [25] that low levels of nitrogen (0.2–3.0 mM) limited cell growth, while higher concentrations (5.0 mM) improved the growth of *C. vulgaris.* Therefore, both concentration and type of nitrogen source in the growth medium influence the growth trends.

3.2. Proteins and Lipids

3.2.1. Proteins

The content of proteins was affected significantly by the growth media and cultivation duration for *Chlorella pyrenoidosa* and by cultivation duration for *C. vulgaris* (Table 2a,b). In both species, the content of proteins increased to the highest level after the second sampling point (eight and five days of cultivation, for *C. vulgaris* and *C. pyrenoidosa*, respectively) and then decreased. This finding is in agreement with other studies showing that the protein content declines with increasing cultivation time [26]. Total N:P ratio in the culture medium in our study was around 17.2 which is higher than the range of 0.5 to 14.7 reported by previous studies [23,26,27]. Effects of N:P ratio on protein accumulation is not clear. He et al. [10] reported that protein content in biomass decreased with an increase in N:P ratio, which was in contrast to the study of Leonardos and Geider [27] who found no relationship between the protein content and the N:P ratio.

The highest levels of proteins in biomass was 65.2% ± 1.3% DW obtained in the 67% ICW experiment with *C. vulgaris*, however, both species showed high levels of proteins, compared to previous reports [10,25,28,29]. Protein content in 100% ICW experiment declined at a slower rate, compared to 34% and 67% ICW experiments for both species, which can be attributed to more available nitrogen in growth media. As mentioned, the biomass productivity in *C. pyrenoidosa* was higher in 100% ICW experiment (Figure 1a,b).

Table 2. Lipid and protein contents of *Chlorella vulgaris* (**a**), and *Chlorella pyrenoidosa* (**b**).

Lipids%DW

	ICW%		
Cultivation time(d)	**34**	**67**	**100**
3	3.51±0.2 [a, x]	4.63±0.0 [a, y]	4.60±0.2 [a, y]
5	5.61±0.1 [b, x]	5.80±0. 3 [b, x]	7.20±0. 1 [b, y]
9	9.70±0.3 [c, y]	9.00±0.1 [c, x]	9.87±0.1 [c, y]
16	17.0±0.1 [d, z]	11.4±0. 4 [d, x]	13.0±0. 4 [d, y]

Proteins %DW

	ICW%		
Cultivation time(d)	**34**	**67**	**100**
3	42.3±2.8 [a, x]	44.8±4.1 [a, y]	42.4±1.1 [a, x]
5	55.1±1.4 [c, x]	54.4±3.3 [c, x]	55.2±1.8 [d, x]
9	49.9±1.0 [b, xy]	47.7±2. 3 [b. x]	51.1±0.9 [c, y]
16	42.0±0.9 [a, x]	44.4±1.3 [a, y]	47.4±2.1 [b, z]

(a)

(b)

Lipids %DW

	ICW%		
Cultivation time(d)	**34**	**67**	**100**
4	2.51±0.2 [a, x]	5.91±0.0 [a, y]	5.75±0.1 [a, y]
8	3.52±0.1 [b, x]	8.80±0.1 [b, y]	8.20±0.2 [b, y]
11	9.30±0.1 [c, y]	10.0±0.3 [c, z]	7.87±0. 3 [b, x]
16	17.6±.0.1 [d, z]	13.1±0.5 [d, y]	10.0±0.3 [c, x]

Protein %DW

	ICW%		
Cultivation time(d)	**34**	**67**	**100**
4	52.3±2.2 [c, x]	60.3±3.7 [c, y]	53.1±1.2 [a, x]
8	55.8±1.0 [d. x]	65.2±1.3 [d, z]	58.2±3.5 [b, y]
11	47.8±1.9 [ab, x]	57.4±2.0 [b, y]	59.1±3.1 [bc, z]
16	46.3±2.8 [a, x]	54.6±2.7 [a, y]	57.8±3.2 [b, z]

(b)

Data are presented as % of dry weight (DW) basis. For each parameter, same letters indicate similar values between cultivation duration (x–z), and ICW percentage (a–d) ($p < 0.05$).

A positive correlation between the proteins content and concentration of nitrogen was already reported by previous studies [10,25,26,28]. Thus, the growth media directly influences synthesis of nitrogen-containing compounds such as proteins, nucleic acids, amino acids, and chlorophyll depending on its level of nitrogen [2]. In a growth media with sufficient available nitrogen, carbon fixated in the photosynthesis process is being used for the protein synthesis. Accumulation of carbohydrates or lipids will start when the nitrogen declines beyond a threshold, which is species-specific [26]. Hence, the protein content in the microalgae depends on the species and is influenced by growth stage, photosynthesis, and available nitrogen. When protein is the target compound in the biomass, higher levels of nitrogen in the growth medium will be required. Use of a priceless effluent like ICW decreases the production cost compared to cultivation with commercial growth media. On the other hand, nutrient removal from the effluent valorizes the resource and makes the production sustainable, as it reduces biological oxygen demand (BOD) of the effluent, which can therefore return safely to the environment without costly effluent treatment procedures. As an example, during 21 days of cultivation and for 100% ICW experiments, more than 99.5% of the ammonia and phosphorus were removed from growth medium. (data are not shown here).

3.2.2. Lipids

Variations in the contents of lipids were significantly influenced by cultivation duration (Table 2a,b) and growth medium ($p > 0.05$), while slightly higher levels of lipids were observed for the 34% ICW experiments followed by 67% and 100% ICW. The highest lipid contents in this study were found after 16 days of cultivation at 17.0% ± 0.1% DW and 17.6% ± 0.1% DW for *Chlorella vulgaris* and *C. pyrenoidosa*, respectively. Lipid accumulation depends on nitrogen availability as the primary factor [2, 10], so in a nitrogen-rich medium, less lipids (and carbohydrates) are being produced by the microalgal cells. Phosphorus is another essential nutrient in growth media, which promotes the growth of microalgal cell and enhances the lipid synthesis. It has been claimed that N:P ratio in a growth media affects the lipid accumulation [10], while other studies [27] have not found any significant relationship between N:P ratio of growth medium and the chemical composition of the algal biomass, especially for the microalgae grown at higher light intensities. Hu [26] suggested that lower N:P ratio enhances lipid accumulation, but the response of all microalgal species is not the same. Lipid synthesis in *C. vulgaris* is reported as a highly complex phenomenon [25]. Chiu et al. [23] found that higher lipid contents could be achieved with more diluted ICW containing lower concentrations of ammonia and phosphorus.

3.3. Fatty acid Composition

Chlorella sp. is a known producer of C18 and C16 fatty acids [24]. In agreement with this, C16:0 and C18:3 *n-3* were the primary fatty acids found in *Chlorella vulgaris* (Table 3a). In the present study, the amount of C16:0 increased during cultivation of *C. vulgaris* in 34% ICW, but this pattern was not observed when higher levels of ICW were used (Table 3a). On the other hand, amounts of C16:0 were higher in the 100% ICW experiment compared to 34% ICW at all time points. Total unsaturated *n-3* fatty acid contents decreased during the cultivation and with increasing concentration of growth media as shown in Table 3a. The same trend was suggested by He et al. [10]. The level of C18:3 *n-3* followed the same pattern as the total *n-3* fatty acids. Overall, the lowest amount of C18:3 *n-3* was observed in *C. vulgaris* cultivated in 100% ICW after 16 days, whereas the highest amount was found at the first sampling point (day 3) in the 34% ICW experiment. Variations in polyunsaturated fatty acids were not the same in *C. pyrenoidosa*, since amounts of C18:3 *n-3* slightly increased during the cultivation time, however this was only significant at 100% ICW (Table 3b).

Table 3. Fatty acid compositions of *Chlorella vulgaris* (**a**); and *Chlorella pyrenoidosa* (**b**). The second row at each table shows the cultivation time (day).

(a)

ICW %	34				67				100			
Fatty Acid	**3**	**5**	**9**	**16**	**3**	**5**	**9**	**16**	**3**	**5**	**9**	**16**
14:0	1.03±0.34	0.57±0.03	0.61±0.07	0.47±0.01	0.81±0.22	1.49±0.62	1.51±0.06	0.29±0.04	0.92±0.04	1.97±0.01	1.60±0.05	0.40±0.01
14:01	0.95±0.00	0.32±0.02	0.53±0.03	0.36±0.01	0.37±0.01	0.27±0.17	0.36±0.04	0.39±0.01	0.53±0.01	0.28±0.00	0.25±0.04	0.38±0.00
15:00	0.20±0.00	0.17±0.01	0.17±0.00	0.11±0.01	0.18±0.01	0.16±0.01	0.14±0.01	0.14±0.00	0.23±0.00	0.25±0.02	0.18±0.01	0.15±0.01
16:0	18.1±2.76	18.4±0.11	20.7±0.04	23.3±0.07	19.1±0.06	19. 0±0.48	17.1±0.01	17.7±0.04	24.0±0.17	23.1±0.05	21.2±0.01	23.1±0.11
16:1 (n-7)	1.88±0.56	1.34±0.04	1.87±0.13	7.15±0.01	2.56±0.20	0.94±0.01	1.62±0.10	17.6±0.01	5.28±0.01	3.58±0.01	1.43±0.01	16.4±0.08
16:2(n-4)	3.46±0.74	4.56±0.01	4.76±0.08	2.51±0.05	4.66±0.04	5.24±0.12	6.18±0.01	1.96±0.01	3.00±0.04	5.90±0.04	6.72±0.04	2.14±0.01
16:4(n-1)	16.2±2.39	15.2±0.01	10.9±0.23	10.9±0.06	16.4±0.05	16.1±0.35	12.4±0.04	10.7±0.11	12.4±0.11	12.7±0.11	9.15±0.11	8.2±0.19
18:0	1.20±0.10	1.03±0.04	1.01±0.01	2.04±0.01	1.14±0.01	0.45±0.02	0.46±0.01	1.50±0.05	1.58±0.00	0.63±0.01	0.85±0.00	3.14±0.02
18:1 (n-9)	5.91±0.88	8.33±0.11	11.4±0.30	13.2±0.08	4.52±0.06	5.53±0.13	7.1±0.08	11.9±0.06	5.82±0.02	4.51±0.01	14.7±0.01	13.2±0.04
18:1 (n-7)	4.01±0.76	3.58±0.01	8.26±0.25	9.37±0.06	3.25±0.01	1.35±0.01	1.89±0.01	7.39±0.01	3.99±0.03	3.16±0.01	2.44±0.00	7.23±0.02
18:2 (n-6)	7.59±1.07	9.73±0.01	10.9±0.17	7.37±0.01	9.68±0.03	12.7±0.31	17.8±0.06	4.72±0.03	9.17±0.02	12.5±0.01	16.7±0.03	6.42±0.03
18:3(n-3)	35. 9±4.88	35.5±0.13	27.6±0.52	22.1±0.10	35.9±0.25	36.2±0.88	33.0±0.08	24.3±0.19	31.7±0.10	31.5±0.11	24.3±0.15	16.7±0.18
20:4 (n-6)	1.07±0.13	0.28±0.06	0.37±0.02	0.20±0.01	0.49±0.02	0.11±0.10	0.05±0.00	0.46±0.01	0.44±0.03	0.13±0.02	0.06±0.00	0.97±0.01
20:5 (n-3)	0.81±0.02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
22:5 (n-3)	1.72±0.25	0.79±0.05	0.77±0.02	0.79±0.01	0.81±0.03	0.21±0.10	0.12±0.01	0.76±0.01	0.83±0.01	0.34±0.01	0.18±0.02	1.44±0.03
∑Sat	20.3±3.20	20.2±0.20	22.5±0.12	25.3±0.10	21.3±0.30	21.1±1.12	19.2±0.08	19.6±0.13	26.7±0.21	25.2±0.07	23.8±0.07	26.8±0.20
∑n-3	38.4±5.15	36.3±0.18	28.4±0.54	22.3±0.11	36.7±0.28	36.5±0.98	33.1±0.08	25.0±0.19	32.6±0.11	31.9±0.12	24.5±0.15	18.1±0.21
∑n-6	8.60±1.20	10.0±0.08	11.2±0.19	7.50±0.01	10.1±0.05	12.8±0.41	17.9±0.06	5.20±0.01	9.60±0.05	12.6±0.04	16.8±0.03	7.30±0.04

Values are given as mean % of total fatty acids (n = 2) ± standard deviation (absolute value).

(b)

ICW %	34				67				100			
Fatty Acid	**4**	**8**	**11**	**16**	**4**	**8**	**11**	**16**	**4**	**8**	**11**	**16**
14:0	0.37±0.08	0.33±0.10	0.49±0.02	0.68±0.10	0.71±0.26	1.06±0.62	0.30±0.02	0.85±0.03	1.50±0.01	0.83±0.25	2.21±0.08	1.79±0.15
14:01	0.10±0.01	0.16±0.01	0.36±0.02	0.52±0.01	0.21±0.01	0.39±0.01	0.79±0.63	0.37±0.05	0.24±0.02	0.52±0.02	0.53±0.02	0.67±0.01
15:00	0.24±0.02	0.08±0.01	0.13±0.06	0.27±0.01	0.25±0.01	0.21±0.00	0.45±0.37	0.16±0.00	0.24±0.03	0.16±0.03	0.30±0.09	0.15±0.02
16:0	13.9±0.09	17.3±0.23	17.8±1.86	16.8±0.23	20.6±0.21	20.7±0.04	17.4±1.22	19.3±0.08	26.1±0.05	21.7±0.26	19.3±2.43	20.3±0.35
16:1 (n-7)	1.54±0.02	0.86±0.28	1.00±0.11	1.22±0.28	0.98±0.29	1.20±0.14	1.13±0.03	1.71±1.07	1.30±0.01	0.97±0.04	1.56±0.01	1.13±0.04
16:2(n-4)	3.24±0.03	2.83±0.04	2.60±0.30	2.65±0.04	5.23±0.07	4.49±0.14	5.75±0.33	5.82±0.05	2.85±0.02	7.04±0.08	4.52±0.49	3.52±0.08
16:3(n-4)	0.11±0.01	0.13±0.02	0.35±0.24	0.00±0.01	0.10±0.01	0.27±0.06	0.26±0.08	0.09±0.00	ND	0.18±0.04	0.58±0.28	0.30±0.13
16:4(n-1)	11.0±0.37	11.0±0.10	10.5±1.35	11.6±0.10	12.2±0.50	12.7±0.31	13.7±0.94	13.9±0.28	10.6±0.09	10.1±0.11	12.7±1.39	12.0±0.24
17:00	ND	0.10±0.01	0.17±0.01	0.10±0.06	0.10±0.14	ND	ND	ND	ND	0.11±0.01	ND	ND
18:0	3.07±0.01	0.85±0.03	1.17±0.17	0.80±0.03	0.95±0.05	1.08±0.01	0.56±0.05	0.44±0.01	1.81±0.01	0.62±0.04	1.37±0.18	0.98±0.06
18:1 (n-9)	15.0±0.18	28.2±0.84	19.3±1.22	18.3±0.84	7.4±0.11	10.6±0.03	10.7±0.91	5.26±0.04	6.51±0.02	7.67±0.01	8.48±0.40	8.03±0.16
18:1 (n-7)	7.98±0.07	2.70±0.03	3.20±0.30	4.52±0.03	3.36±0.22	2.50±0.02	2.36±0.21	1.80±0.07	6.89±0.00	2.02±0.01	3.14±0.04	3.07±0.03
18:2 (n-6)	12.3±0.03	10.4±0.19	11.9±1.29	11.7±0.19	14.9±0.06	11.6±0.38	13.3±1.06	15.4±0.18	9.8±0.01	16.8±0.20	11.0±1.17	11.8±0.28
18:3(n-3)	30.5±0.27	24.2±0.08	27.7±3.08	29±0.08	32.0±0.33	29.1±0.84	32.0±2.47	33.3±0.10	31.3±0.11	30.1±0.28	30.8±3.36	33.9±0.86
20:1 (n-9)	ND	ND	ND	ND	ND	0.16±0.01	ND	ND	ND	ND	0.17±0.01	0.09±0.01
20:4 (n-6)	0.25±0.03	0.14±0.01	0.96±0.14	0.39±0.01	0.19±0.02	0.87±0.08	0.24±0.01	0.26±0.04	0.25±0.01	0.24±0.02	0.79±0.09	0.47±0.01
20:5 (n-3)	ND	0.23±0.02	0.60±0.30	0.65±0.02	0.33±0.01	1.32±0.04	0.33±0.03	0.45±0.16	ND	0.33±0.01	1.11±0.06	0.71±0.01
22:5 (n-3)	0.43±0.02	0.32±0.05	1.67±0.22	0.83±0.05	0.33±0.02	1.62±0.07	0.44±0.04	0.76±0.12	0.53±0.04	0.45±0.03	1.32±0.12	0.94±0.10
∑Sat	17.6±0.18	18.7±0.43	19.7±2.09	18.5±0.43	22.6±0.66	23.1±0.67	18.7±1.66	20.8±0.09	29.6±0.10	21.4±0.59	23.2±2.78	24.2±0.57
∑n-3	31.0±0.29	24.7±0.16	29.9±3.59	30.4±0.16	32.7±0.33	32.0±0.95	32.8±2.53	34.5±0.69	31.8±0.15	30.92±0.29	33.2±3.54	35.5±0.98
∑n-6	12.5±0.06	10.5±0.19	12.9±1.44	12.1±0.19	15.1±0.06	12.4±0.46	13.6±1.07	15.6±0.22	10.1±0.02	17.0±0.22	11.8±1.27	12.3±0.28

Values are given as mean % of total fatty acids (n = 2) ± standard deviation (absolute value).

The same pattern was observed for the total *n-3* contents, and variations in C16:4 *n-1* were also different in the two *Chlorella* species. In *Chlorella vulgaris*, amounts of C16:4 *n-1* declined with the cultivation duration, unlike the variations of this fatty acid in *C. pyrenoidosa* biomass. Similarly, higher amounts of C20:5 *n-3* and C22:5 *n-3* were found in *C. pyrenoidosa* compared to *C. vulgaris*. Similar, the variations in C18:1 *n-9* in *C. pyrenoidosa* was higher compared to *C. vulgaris,* with higher values in lower concentrations of ICW. These results confirm previous studies [10,27], showing that monounsaturated fatty acids accumulated when the nitrogen concentration in the growth medium was decreased [27].

3.4. Tocopherols

Tocopherol composition in both *Chlorella* species mostly include α-tocopherol as reported previously [22]. Contents of α-tocopherol increased during the cultivation, but the patterns were not the same at all concentrations of ICW in both species. The highest content of α-tocopherol was observed in 34% ICW experiment after eight days in *Chlorella vulgaris* (Figure 2a), whereas the 67% ICW experiment with *C. pyrenoidosa* resulted in the highest amount of α-tocopherol after 16 days (Figure 2b). In general, the concentration of α-tocopherol was higher compared to previous reports for *Chlorella sorokiniana* cultivated in wastewater [22]. To our knowledge, variations of α-tocopherol in *C. vulgaris* and *C. pyrenoidosa* during the growth was not reported in previous studies.

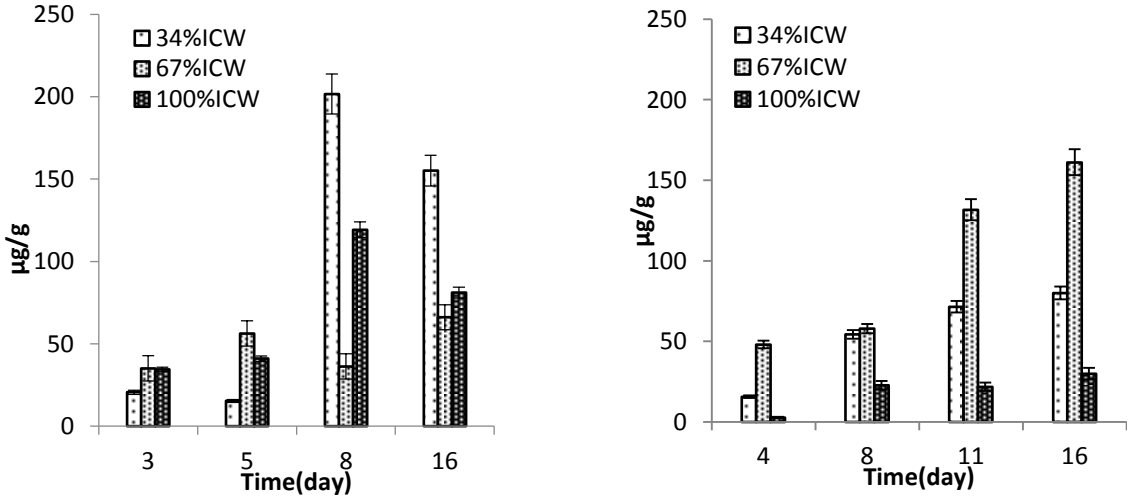


Figure 2. Contents of α-tocopherol in *Chlorella vulgaris* (**a**); and *Chlorella pyrenoidosa* (**b**), during the cultivation on ICW. The error bars represent the upper and lower values.

3.5. Amino Acid Composition

Amino acid composition in both *Chlorella* species includes arginine, glutamine, lysine, asparagine and leucine as principal components (Figure 3a,b). Amino acid composition in all microalgae is similar [4,30–32], but it can be influenced by variations in environmental conditions and growth medium composition. Amino acid profile of *Chlorella pyrenoidosa* in this study includes lysine, methionine, threonine, tryptophan, histidine, leucine, isoleucine, valine and phenylalanine. During cultivation, contents of glutamine, asparagine and lysine decreased in both species. Total amino acid contents also decreased, which correlates with variations in protein contents in the samples. Fish meal has always been the primary source of protein in the formulation of fish feed [33], partly due to its high protein content (ca. 70% DW) and excellent amino acid profile [34]. The continuous increase in both demand and price of fish meal emphasizes the need to find sustainable alternative resources. The biomass from the studied *Chlorella* species includes proper amounts of protein (ca. 58% DW for *C. pyrenoidosa*) and promising amino acid composition, which makes them a promising fish feed ingredient.

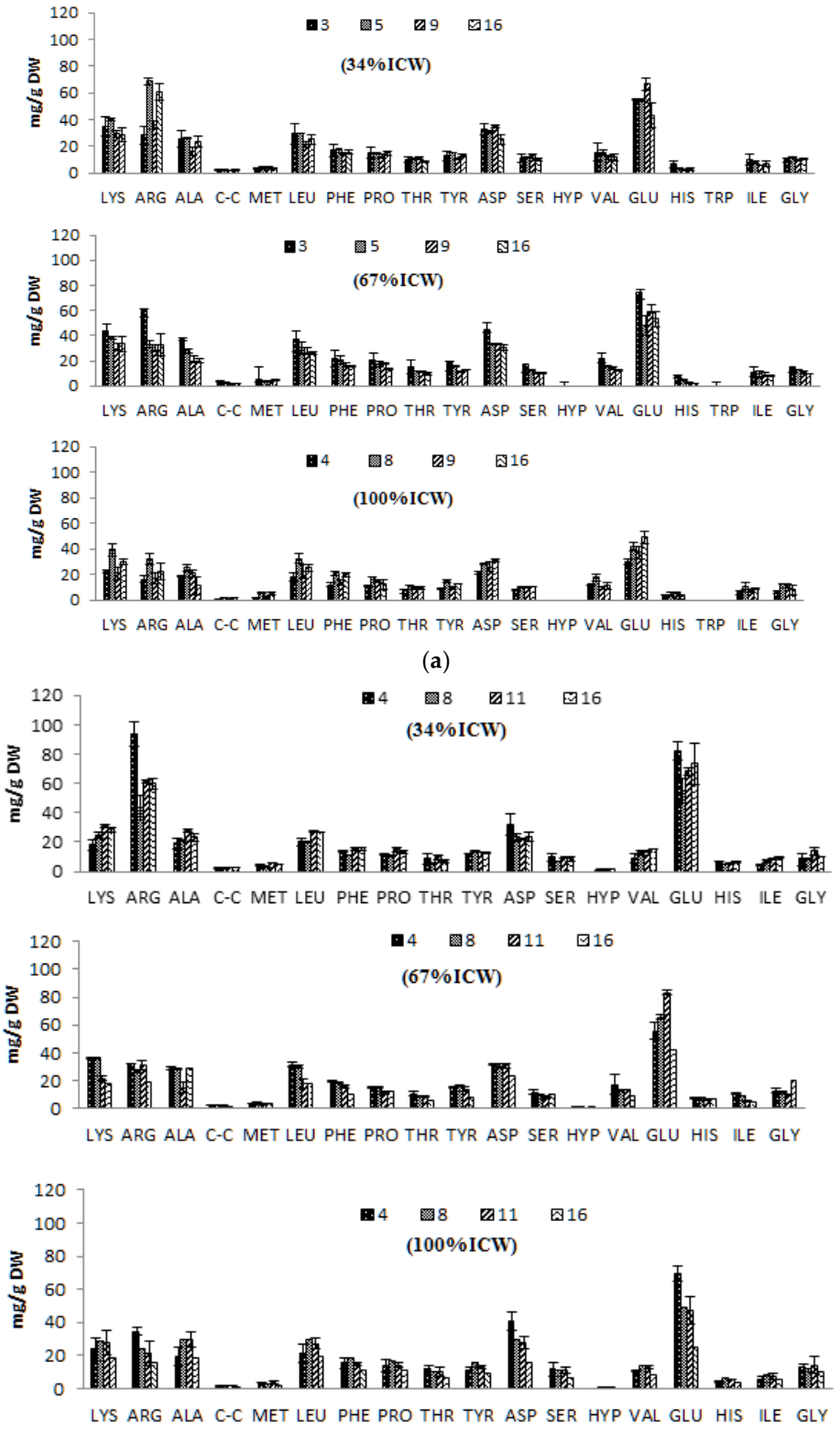
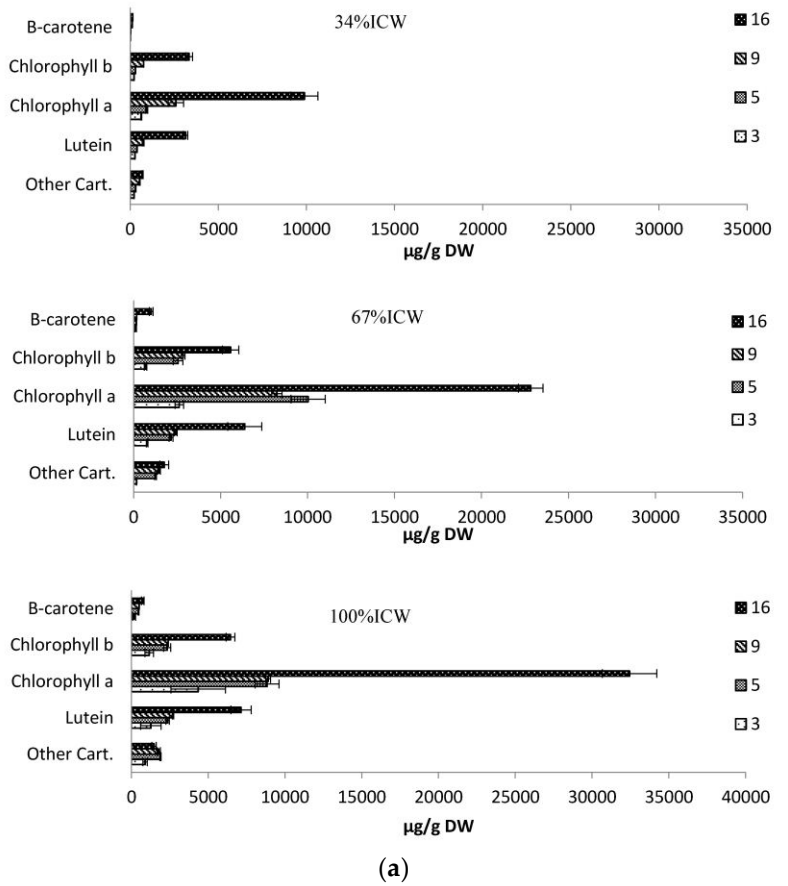


Figure 3. Amino acid composition in *Chlorella vulgaris* (**a**); and *Chlorella* pyrenoidosa (**b**), during the cultivation in ICW. The error bars represent the standard deviation. LYS;Lysine, ALA;alanine, ARG;arginine, LEU;leucine, C-C; cysteine, MET;methionine, PHE; phenylalanine, PRO;proline, THR;threonine, TYR;tyrosine, ASP; asparagine, SER;serine, HYP; hydrocy proline, GLU;glutamine, VAL;valine, HIS;histidine, ILE;isoleucine, GLY;glycine

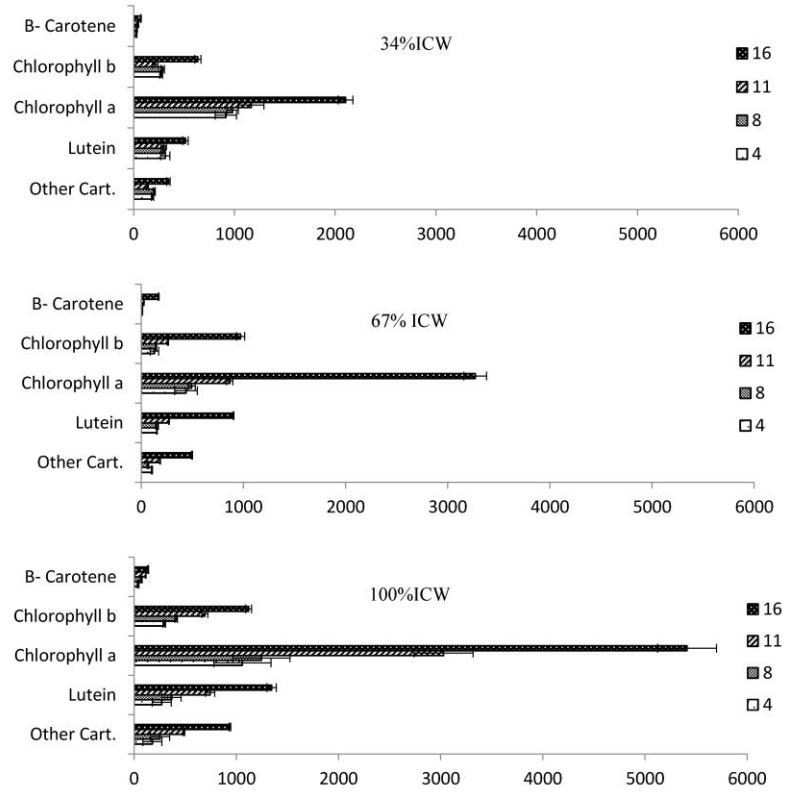
Animal source of amino acids such as fish meal contain a good balance of essential amino acids, but plant proteins such as soybeans are known as poor-quality as they lack some amino acids [4,35]. All fish require ten essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine [36]. Amino acid composition of Chlorella sp. can be represented as a rich source of essential amino acids for the formulation of fish feed.

3.6. Pigments

The pigment composition in *Chlorella* species includes carotenoids and chlorophylls (Figure 4a,b). Lutein was the main xanthophyll in both species. Pigment production increased during the cultivation. In both species, a positive correlation between the pigment content and level of ICW was found. *Chlorella vulgaris* produced very high concentrations of chlorophylls and carotenoids with a plateau of 7141 ± 661 μg/g DW lutein and 32,444 ± 1772 μg/g DW chlorophyll a, at day 16 for microalgae cultivated on 100% ICW (Figure 4a). These amounts are approximately six times higher than for *C. pyrenoidosa* (Figure 4b) at 1345 ± 45 μg/g DW and 5411 ± 145 μg/g DW for lutein and chlorophyll a, respectively. The highest amount of β carotene (1013 ± 107 μg/g DW) was observed for *C. vulgaris* at day 16 in the 67% ICW experiment, whereas the highest concentration of β carotene (171.8 ± 3.9 μg/g DW) was observed at day 16 in the 34% ICW experiment for *C. pyrenoidosa*. The pigment production pattern was the same for both species. This showed that concentration of nitrogen in growth media influenced the accumulation of chlorophylls as previously reported [9,10,25]. High concentration of lutein (approx. 7.4 mg·L^{-1}) was reported in *Chlorococcum citriforme* SAG 62.80 cultivated in optimal conditions by Del Campo et al. [36] He et al. [10] found that pigments reached their highest levels (0.2%–0.5% of the microalgal biomass) at very elevated levels of NH_4^+-N (210 mg·L^{-1}) and also reported a positive correlation between concentration of pigments and levels of NH_4^+-N. Biosynthesis of carotenoids in microalgae is species dependent and highly affected by growth stage and growth conditions [36] Nitrogen is essential for the production of chlorophyll [2]. However, the accumulation of high levels of lutein and other carotenoids can be justified with the fact that optimal growth conditions, as well as sufficient nutrients, provide enough energy for the production of lutein and other carotenoids. The highest lutein concentration of approx. 0.7 pg·cell^{-1} has been reported in early stationary stage [35] for *Chlorococcum citriforme* SAG 62.80. Cordero et al. [37] reported a marked (from 1.4 to 3.2 mg·g^{-1} dry weight) increase in volumetric and cellular lutein accumulation in *C. sorokiniana*, when the content of nitrate in growth media rose from 10 to 40 mM. The study also reported that random mutagenesis increased the cellular lutein content up to 7.0 mg·g^{-1}.



(**a**)



(**b**)

Figure 4. Pigment composition in *Chlorella vulgaris* (**a**); and *Chlorella pyrenoidosa* (**b**), during the cultivation on ICW. Sampling day is noted as 4, 8, 11, and 16, distinguished by the different patterns. Other carotenoids: zeaxanthin, lutein derivatives, canthaxanthin and violaxanthin. The error bars represent the standard deviation.

The high concentration of carotenoids (ca. 7 $mg \cdot g^{-1}$ DW lutein) in the produced biomass makes it suggestible as a promising ingredient in the formulation of fish feed [5], foods, health care products or cosmetics.

4. Conclusions

Cultivation of *Chlorella vulgaris* and *C. pyrenoidosa* is feasible using different percentages of industrial process water, and *Chlorella pyrenoidosa* was even able to grow in 100% industrial process water and produce elevated levels of biomass. Protein and pigment contents were enhanced by higher percentages of industrial process water in growth media, and protein contents were higher in *C. pyrenoidosa* than in *C. vulgaris,* while *C. vulgaris* produced extremely high amounts of both chlorophylls and carotenoids.

Resulting biomass with moderate lipid content and high levels of protein and carotenoids can be presented as a valuable ingredient for the aquatic feed industry. With further extraction and fractionation, biomass could also be a rich source of protein and lutein for the e.g., food and cosmetic industry. Future upscaling to industrial scale could lead to sustainable, environmentally friendly valorization of a priceless resource of waste water. Beside this, bio-utilization of nutrients such as nitrogen and phosphorous reduces the biological oxygen demand of the waste water, saving on costs of waste water treatment before release to the environment.

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Conflicts of Interest: The authors declare no conflict of interest.

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Paper 5

Downstream processing of microalgae with particular focus on its application as a fish feed ingredient

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Review article

Downstream processing of microalgae with particular focus on its application as a fish feed ingredient

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Abstract:

The microalgae bio industry is growing very fast. A large number of studies have demonstrated several applications for microalgae biomass and its bioactive compounds during the past decades. Now it is becoming increasingly evident that algae biomass is a promising alternative resource of protein, carotenoids and essential fatty acids such as eicosapentaenoic acid. Hence, microalgae biomass is an alternate promising resource of bioactive compounds for the formulation of fish feed. Because of a very low concentration of microalgae in growth medium, downstream processing of microalgal biomass is known as a major and costly component of production. Downstream processing of microalgae is application specific. An efficient and cheap processing concept is required when the resulting biomass is intended to be used as a fish feed ingredient. This review describes different methodologies available for the harvest and dewatering and evaluates their feasibility for the production of microalgae as a fish feed ingredient.

Introduction:

Microalgae are a diverse group of autotroph microorganism which has gained increased interests during the last decades, in both research and commercial sections. Microalgae are considered an abundant, sustainable source of biochemicals with high biomass productivity and the particular ability for conversion of carbon dioxide to oxygen and bioactive compounds. It has been estimated that microalgae produce more than 15000 metabolites [1]. Microalgae can also grow on effluents from different sources and reduce their biological oxygen demand by conversions of nutrients such as ammonia and phosphorus to protein, lipids, vitamins, pigments and many other valuable compounds. It has been estimated that there are more than 800.000 microalgae species, among them 35000 species have been identified [2]. At present, only a few species such as *Chlorella, Dunaniella, Arthrospira, Aphanizomenon, Haematococcus, Crypthecodinium* and *Shizochytrium* are commercialised [3]. The cell size of microalgae highly varies between a few microns, such as *Nannochloropsis* sp., to a few hundred microns for *Arthrospira* sp. [4]. Biochemical composition of microalgae highly varies and depends on several factors such as species, culture age, growth media composition and environmental parameters such as light and temperature. In general, they include 8-70% protein, 4-70% carbohydrates, and 2-50% lipids [5- 7]. Microalgae biomass also includes nutraceuticals such as carotenoids, vitamins and coenzyme Q10. The potential of microalgae biomass as a source of bioactive compounds and energy has been demonstrated in numerous studies [1]. Microalgal applications can be categorized into two broad areas as fuel and non-fuel. Fuel applications include biodiesel, methane, ethanol, and hydrogen. Non-fuel applications are nutraceuticals, food and feed, pharmaceuticals, wastewater treatment and pollution control, cosmetics, and chemicals such as biopolymer and lubricants [8]. The design of downstream processing is application specific. There are several challenges in the commercial processing of microalgae [9] including, management of large scale cultivation, separation of microalgae from the growth media (harvest), and finally dewatering of the biomass [10]. These challenges need to be addressed to ease the application and to extend the shelf life of the product [2]. Microalgal production is not still economical [11], so processing setup should be designed to improve the economy of production. The review gives a general overview of reported technologies

for harvesting and drying methods applied to microalgae with particular focus on the applications for microalgae intended for aquatic feed.

Harvest of microalgae

Harvest is the main part of the downstream process and plays a major role in the energy consumption of the whole process and quality of the biomass [12]. The concentration of microalgae biomass in growth medium is very low (0.1 to 5 g L^{-1}) [9, 13]. So a particular procedure is required to remove the water from biomass without adverse effects on the quality and with minimum possible required energy. The small size, negative surface charge and mobility of microalgae make the process complex [10, 14]. There is not a globally accepted harvest technique for microalgae biomass [4, 8, 9, 12, 14- 15]. Several methods for the harvest of microalgae have been presented as filtration, flocculation, flotation, and centrifugation [8-10]. A combination of any of these techniques may also be used [3, 9]. For the production of low-value biomass (such as fish feed), an economically feasible technique is highly required [15].

Flocculation

Chemical flocculation

Chemical flocculation involves the addition of chemicals such as aluminium oxide, iron (III) chloride, cationic polymers, chitosan and surface active agents to the microalgae cultures [9, 11, 14, 16]. Resulting algae flocks ease the subsequent separation by filtration, centrifugation or sedimentation. Sedimentation by raising the pH to levels around 12 (autoflocculation) was also suggested in some previous reports[9]. This method reportedly required large quantities of caustic soda or lime, which negatively affects the quality of microalgae biomass. The chemical composition of microalgae biomass might be influenced at higher pH values, e.g. proteins and lipids. The remaining caustic soda or lime increase the ash content in the biomass, which decreases its nutritious quality. Choi et al. [16] demonstrated that eggshells could be used as a bio-flocculant in the harvesting of *Chlorella vulgaris*. The report showed that eggshell at the optimized conditions acts as a biopolymer, which agglomerates the microalgae cells. In a method described by Lim et al. [17], *Chlorella* sp. was harvested from growth media with a high efficiency (99%) by

low-gradient magnetophoretic separation. In this method microalgae cells were bound to iron oxide nanoparticles, in the presence of cationic polyelectrolyte as binding agent and then separated in a magnetic field. The study showed that the magnetic nanoparticles shall have excellent colloidal stability before binding to the microalgae for an efficient harvest process. Seo et al. [18] suggested oxidised dye wastewater as a potential and cheap coagulant for microalgae harvesting. However, such coagulant has limited applications.

Bio-flocculation

Application of bio-flocculation on microalgae harvest was reported in numerous studies [2, 11,20-22]. Application of bacteria such as *Paenibacillus* sp. AM49 for the separation of *Chlorella vulgaris* has also been demonstrated by Oh et al. [19]. In another report [21] bacterium *Burkholderia cepaciahas,* which was cultivated in parallel with the microalgae, was used as a bio-flocculation agent of two microalgae *Scenedesmus* sp. and *Chlorella vulgaris.* The reportedly better response of *Sedenesmos* sp. compared to *C. vulgaris*, demonstrates the specificity of the process to different species. The harvest efficiency is therefore influenced by several factors such as microalgae size, shape, and culture density.A fungus pelletization assisted bio-flocculation method for harvesting microalgae has also been reported [23]. When resulting biomass was aimed to be used as food/feed ingredient, application of chemicals such as polymers, surface active compounds, symbiosis microorganisms and inorganic salts are not favored.

Ultrasonic assisted flocculation

Some studies have indicated that ultrasonic irradiation can coagulate the microalgae cell and as a result, assist in the separation of biomass [24- 26]. Separation efficiencies higher than 90% were reached for different microalgae [24]. The effectiveness of ultrasonic irradiation in microalgae cell rupture was shown in numerous studies [e.g. 27], but its potent adverse effects on the cell wall and biochemical composition of harvested algae have not been demonstrated. In a recent study, an on-chip acoustofluidic platform was used for selective harvest of microalgae species in a mixed culture of microalgae and other particles such as contaminants [26]. The method was reported to be rapid, non-invasive and energy-efficient. In this technique, the combination of

microfluidics and acoustophoresis was used and resulted in 90.1% recovery of the target microalgae (*Euglena gracilis*) and 98.3% cell viability.

Flotation

Flotation is another method of harvest in which small bubbles created by electrolysis or pressure fed to the suspension attach to the cell walls and carry them to the top layer. The resulting top layer can be separated from the growth media by different methods such as skimming [11, 28-31]. This technology has been used to some extent and demonstrated acceptable results [32], particularly in combination with coagulants [9-11]. At present, flotation methods are categorized as dissolved air flotation (DAF) with bubble diameter <100 mm; dispersed air flotation, when bubble diameter is 100–1000 mm; electrolytic flotation and ozonation-dispersed flotation. DAF reportedly is the most efficient and widely employed [11]. Coward et al. [30] reported that a foam floatation method using cationic cetyltrimethylammonium bromide (as foaming agent) required less energy compared to other harvest technologies.

Electrical harvest methods

In these methods, an electrical field is applied to the growth medium and negatively charges the microalgal cells, so microalgae precipitate on the electrodes or at the bottom of the tank [12]. Harvesting of marine microalgae by this method was more expensive than freshwater microalgae because of higher conductivity [33]. Type and material of the electrode highly influence the efficiency of the harvest process. Aluminum electrodes reportedly showed higher efficiency compared to iron electrodes [11]. Despite the positive results, which were found in several studies, electrical methods are energy consuming, and too expensive to be used in large scale [12]. Before being commercialized, adverse effect on the quality of harvested biomass should also be explored.

Screening and filtration

Micro strainers and screen filters are the two main types of screens, which have been used previously for microalgae harvesting [10]. Micro strainers consist of rotating drum filters covered with a stainless steel or polymeric micromesh (mesh size ranging from 15 μm to 64 μm) and a

backwash system [12]. The drum filter is being submerged in the filtering medium and let the substrate flow through the open end of the drum and then the medium is filtered through the screen, and particles remain on the filter. Different ways can be utilized to separate the biomass from the filter Microalgae size and dry matters of the medium are critical factors in the application of this technique. Large microalga species can be separated faster and cheaper. High-density cultures cause clogging of the strainer surface, and this decreases the efficiency [12].

Pressure Filtration

Filtration is an inexpensive method of harvest. Plate and frame or pressure vessels such as leaf filters have been used for microalgae [10, 34]. Filtration requires a driving force (gravity, vacuum, pressure, or centrifugal force) to provide the pressure needed to move the fluid through the filter [15, 12]. Biomass can be collected on the surface or within the filter medium, which is required to enhance the filtration and to retard clogging of the filter medium [12, 31]. During the filtration, the thickness of separated microalgal deposits on the filter gradually increases and as a result, resistance increases and flux decreases, which finally causes fouling [11]. Conventional frame and plate filtration are just applicable to the large sized microalgae such as *Arthrospira* sp. or *Coelastrum proboscideum* [31]. However, separation of microalgae pastes from filter aid, which is a common use, is difficult.

Micro - ultra membrane filtration

In general, the membrane is a selective barrier between two phases, which perform the separation using a driving force. Membrane-based processes are being more popular because of their diverse applications and several advantages [15]. Two common operation mode of membrane filtration are dead-end and cross-flow. Dead end mode normally is applied to the large microalgae like *Arthrospira* and provides higher concentrations of the paste [12]. Cross flow filtration is the method of choice when the harvest of small size microalgae is considered [12]. In this method, the medium flows tangentially across the membrane and the resulting biomass paste (retentate) is being circulated, preventing the membrane from fouling [11, 15]. This method is more appropriate for the harvesting of microalgae with smaller size due to minor fouling problems but will result in

lower biomass concentration compared to centrifugation. In theory, microfiltration membranes can filter particles in the range of 0.025 μm to 10.0 μm. Hence, microalgal cells could be harvested by this approach. Membrane filtration is not commonly applied in large scale yet. There are many fibrous matrices with proper pore size range, but only a membrane filter with a narrow pore size range can ensure quantitative retention. Membrane filters can be used for final filtration or prefiltration. The depth filter is employed in clarifying applications or as a guard filter to prolong the membrane's working time. Application of micro/ultrafiltration for the harvest of microalgae has already been indicated in numerous studies during the past decades[15, 35-41]. All of the previous studies focused on the harvest for biofuel application. The majority of the membrane materials used in previous studies on microalgae are polymeric [15]. These organic phases reportedly include; polyvinylidene fluoride (PVDF), cellulose acetate (CA), polytetrafluoroethylene (PTFE), polypropylene (PP), poly(ether sulfones) (PES), polyvinyl chloride (PVC), polyacrylonitrile (PAN) and polyethylene terephthalate (PET). Application of ultrafiltration for single step harvest and concentration of *Scenedesmus quadricauda* to 15% W/V has been shown by Zhang et al. [37]. In another study, harvest efficiency of *Cylindrotheca fusiformis* and *Skeletonema costatum* microalgae, by crossflow ultrafiltration flat sheet membrane and dynamic filtration modules were evaluated [42]. The study showed almost twice higher microalgae harvest efficiency for dynamic filtration system compared to cross flow ultrafiltration, both at constant concentration and in concentration mode for two microalgae species. Dewatering efficiency of *Nannochloropsis* sp. was evaluated by a two-step membrane filtrations harvesting process in which, the polymeric hollow fiber and tubular ceramic membranes have been used as the first and second phase [38]. In this study, the filtering ability was improved by using a coagulant ($FeCL_3$). Hwang et al. [40] showed that hydrophilic polyvinyl alcohol coating of the membrane improves the filtration rate and maximum flux. The applicability of submerged microfiltration for harvesting *Chlorella vulgaris* and *Phaeodactylum tricornutum* has been investigated by Bilad et al. [43]. The study suggested that submerged microfiltration for algal harvesting is economically feasible. Buckwalter et al. [44] suggested that forward osmosis can be an energy-saving step in dewatering freshwater microalgae if conditions are controlled to prevent leakage.

In general membrane technology is cheaper than centrifugation but the concentration of resulting biomass still is low (ca. 20-30 g/L) for an efficient and feasible drying, if this is required for applications such as fish feed.

New trends:

Liqtech Silicon Carbide Ceramic (SiC) microfiltration membranes

The Danish company Liqtech international A/S, patented SIC membrane been successfully tested for the harvest and pre concentration of various microalgae including *Dualiella* sp., in both laboratory and large scale [45]. The Technology showed the possibility of harvest and pre-concentrates of the algae to a dry weight percentage of 7-11 %(W/V) for *Dunaliella* sp.

Algae Venture's harvesting technique

A new harvest technology which claims to reduce energy consumption by utilizing surface physics and capillary action has been introduced by Algae Venture Company in the US. The system includes two belts which move in opposing directions. The algae suspension is fed to the top belt; water penetrates through the belt, while the solid remains on top. Water removal occurs via capillary action. The algae are dewatered without pressure. Drying can occur through evaporation, or with a stream of waste heated blowing air [46].

Trilobite microfluidic chip

A new microalgal harvest method using a trilobite based microfluidic chip was presented by Hønsvall et al. [47]. The chip with a 5 μm gap limit was tested on microalgae *Rhodomonas baltica*, *Chaetoceros* sp. and *Thalassiosira weissflogii*. The technology appears to be a promising for hard to harvest microalgae such as microalgae with small size or repelling properties. This technique has not tested in large scale.

Centrifugation

Centrifugation is probably among the most favored methods of the harvest of microalgae [4, 9-11, 31, 32, 48]. This approach has already been used commercially for the up-concentration of

harvested microalgae suspension. Centrifugation is fast but expensive, so normally it is being used for high-value products of microalgae such as food/pharmaceutical ingredients [12]. The separation efficiency depends on the particle size and the difference in the density of microalgae and growth medium [12]. Centrifugal power directly influences the separation efficiency. Separation efficiencies of more than 95%, 60%, and 40% have been obtained at 13000 g, 6000 g, and 1300 g, respectively and examined for nine microalgae species [49].

Centrifuges have two main applications; for .sedimentation of solid, or filtration of liquids. There are different designs of centrifuges, which in general are categorized as self-cleaning, disc-stack centrifuge, nozzle discharge centrifuge, decanter bowl centrifuge, and hydro-cyclones [4, 31, and 50]. Disc-stack centrifuges are one of the most common systems, which are widely used for the harvesting of microalgae [4, 49]. A disc stack centrifuge includes a shallow rotating cylindrical bowl with several conical metal discs. The substrate is fed to the center of the discs and under centrifugal force, the heavy phase moves to the sides of the discs, and the light phase moves downward to the center tube, and then is directed to the outlet. Disc-stack centrifuges reportedly can be utilized for the harvest of algal cells from growth medium [4]. Residence time was shown to be important for the separation efficiency and can be controlled by the flow rate [49]. It has been claimed that decanter centrifuge has lower microalgae recovery efficiency than disk stack centrifuge, but can provide a more efficient output, albeit at the expense of a high energy consumption [31]. Commercial types of disc-stack centrifuges are developed by different companies and are available in the market [9]. The main application of nozzle discharge centrifuges is final upconcentration or pre-concentration, while bowl decanter centrifuge just is suitable for final upconcentration. [50]. Hydro-cyclones are not appropriate for the application of microalgae, because of their poor efficiency [51].

The hydro cyclone

Hydrocyclone has the same function and principle as cyclones and include a conical part connected to a cylinder, albeit with a different design. Feed flows tangentially into the cylindrical section and develops a strong swivelling motion trough the conical part. Fine particles move up to the outlet with the clarified liquid, as the light phase and the heavy phase, consisting of algae

biomass, settles down the conical part and can be discharged via the underflow [10]. The efficiency of hydro cyclone algal separation is reportedly low and therefore the hydro cyclone is not appropriate for the application of microalgae [9, 10, and 51].

Solid bowl decanter centrifuges

Decanters are categorized as horizontal or vertical decanters. The horizontal decanter is characterized by a straight conical bowl, which contains a screw conveyor that rotates in the same direction. Feed slurry enters at the center and is centrifuged against the bowl wall. In horizontal decanters, settled solids are moved by the screw conveyor to one end of the bowl before discharge, while separated water phase forms an inner concentric layer, which flows over an adjustable dam plate and is discharged out of the centrifuge. The helical screw conveyor that pushes the deposited slurry operates at a higher rotational speed than the bowl. The solid bowl decanter centrifuge has been recommended for use concurrently with polyelectrolyte coagulant to increase the efficiency [34].

Nozzle-type centrifuges,

In nozzle type centrifuges, the heavy phase is continuously discharged via nozzles located around the periphery of the bowl and subsequently collected in the bowl. In this type, the shape of the bowl provides sufficient storage volume and facilitates the cake to eject out [10]. Nozzles are arranged around the bowl and are directed tangentially backwards to the direction of rotation. The number, size and design of the nozzles are optimized to avoid cake buildup and to obtain reasonably concentrated algal biomass. Golueke and Oswald [52] studied the effects of nozzle diameter on flow rate, algae removal efficiency, and resultant slurry concentration. The nozzle-type centrifuge was able to produce biomass with higher concentrations (e.g. 30-35% dry matter), compared to other harvest techniques, albeit with higher energy consumption.

Despite efficient harvesting, centrifugation cause cell rupture under high shear rates, which is a disadvantage when great value biochemical such as carotenoids or polyunsaturated fatty acids are lost. The technique can be utilized to harvest most of the microalgae, but in most cases a previous harvest might be required for an efficient process. When the concentration of dry matter

in growth media is very low, the energy consumption for the separation of algae is very high [53]. In cases that microalgae biomass aimed to use as a medium / low-value products such as fish feed ingredient, then the energy consumption would be a critical factor. The combination of this technique with the other methods in set ups such as; flocculation & sedimentation &centrifugation or microflotation & centrifugation reportedly reduces the overall process cost to a low rate [9]. Table 1 summarizes the details of different harvest and up-concentration methods, their recovery, pros and cons, and the microalgae which were studied as reported in the literature, offering *Chlorella* sp., as the most studied microalgae species

New trends- Evodus dynamic settler

Evodos has presented a new technology of harvesting called dynamic settling method. The Evodos equipment consists of spiral flow moving plates, providing a gentle but effective centrifugal force and accelerating the gravity (max. 3000 g). Harvested algae can be separated manually after opening the system. The Evodos system is commercialised now and successfully tested [54], but there is no data regarding the system efficiency or quality of the final product in the literature.

Drying

Drying is the last part of microalgae processing in which, the moisture content is decreased to less than 10 % [10]. Drying is also the most expensive part of downstream processing, and constitutes 75% of the processing cost [10, 34]. Further processing of algae paste with 25 % dry solids may not be feasible for low-value applications such as biofuel, but for several applications including food and feed, a dry biomass with reasonable shelf life is required. Application, storage, and transportation of dried biomass is more economical than wet biomass paste. There are different drying techniques for drying of microalgae, which highly differ in the extent of capital investment, energy requirements [10, 34] and quality of the final product. Selection of drying technique depends on the capacity and quality of final dried biomass [10, 31, 34]. Many different drying methods have already been applied to microalgae including sun drying, fluidised bed drying, spray drying, drum drying, incinerator drying, cabinet drying and flash drying.

Table 1. Different harvesting technologies applied to various microalgae species. N/A; information is not available.

Harvest method	Microalgae	Recovery	Advantages	Disadvantages	Reference
Chemical & auto Flocculation	*Chlorella vulgaris, Choricystis minor, Cylindrotheca fusiformis, Neochloris sp., Nannochloropsis salina, Neochloris oleoabundans, Dunaniela salina, Chlorococcum sp., Chaetoceros calcitrans, Chaetoceros muelleri Chlorella, Isocrysis galbana, Pavlova lutheri, Tetraselmis sp., Thalassiosira pseudonana, Phaeodactylum tricornutum*	67-99 %	Simple, require low energy,	Requires chemicals (sometimes toxic), and subsequent separation techniques. Requires more space. Could not be used as a feed ingredient when non-feed grade chemicals are used.	[9], [14], [11],[17],[18],[22]
Bio- Flocculation	*Botryococcus braunii, Scenedesmus quadricauda, Selenastrum capricornutum, Anabaenafios-aquae, Microcystis aeruginosa, Nannochloropsis oceanica, Pleurochrysis carterae, Chlorella sp., Pediastrum sp., Phormidium sp., Scenedesmus sp., consortium, Neochloris oleoabundans*	>83%	Non-toxic, low processing cost particularly for microalgae cultivated on waste water	Risk of contamination, variation in chemical composition, may require additional ingredients in the growth media	[11], [12], [16], [19], [20], [21], [23], [40], [22]
Ultrasonic assisted flocculation	*Monodus subterraneus, Euglena gracilis, Mychonastes aff. jurisii TKAC1031*	83 ± 12%	selective flocculation for some microalgae	Risk of cell rupture	[24], [25], [26]
Flotation	*Chlorella sp. Dunaliella salina, Tetraselmis sp., Microcystis aeruginosa, Asterionella formosa, Scenedesmus quadricauda, Melosira sp.,*	>85%	Requires low space , energy and processing time	Requires chemicals (sometimes toxic) and subsequent separation techniques. Not suitable for marine microalgae	[28], [11], [31], [10], [9], [30]
Electrical harvest methods	*Dunaliella salina, Chlorella sp., Microcystis aeruginosa,*	45-100 %	Efficient for fresh water algae	Requires relatively high energy. The emission of unwanted substances and H2 gas. Higher energy required for marine algae	[22], [12], [32]
Screen filtration, Pressure Filtration	*Arthruspira sp., Coelastrum proboscideum , Scenedesmus sp., Oscillatoria sp.*	< 90%	Simple, low maintenance and capital price	Suitable for very big microalgae cells, clogging.	[43], [12], [31]
Membrane filtration; Micro and ultra-filtration, reverse osmosis	*Chlorella sp., Phaeodactylum sp., Scenedesmus quadricauda , Nannochloropsis oculata, Nannochloropsis gaditana*	90-100%	Efficient, requires low energy. Low shear stress	clogging problems, High maintenance and operation costs for cleaning and changing of membranes, require higher pumping energy	[35], [36],[37], [38], [43], [69], [40], [44], [15], [42]
Centrifugations	*Scenedesmus sp., Coelastrum proboscideum Nannochloropsis sp. , Chlorella sp., Dunaliella sp.,*	90-100%	Fast, efficient, suitable for various cell sizes, even small microalgae.	High shear stress(disk stack), high capital and operation costs, not suitable for microalgae with thin cell walls	[10, [49], [12], [9], [48], [50], [51], [11], [31], [4]

The majority of the algal drying methods are reportedly originating from regular sewage sludge drying systems [10]. Some new and specific drying techniques such as pulse combustion [55] and ocean chill drying have also been introduced [56].

Sun (solar) drying

Sun (solar) drying is probably one of the oldest and the most simple method of food dehydration, which is still popular in many developing countries [9-10]. This technique has two

types of direct or indirect method. Indirect method uses the hot air heated up by solar energy as drying agent [34; 31], which prevents the product from overheating, which normally occurs in the direct method. Sun drying has been suggested as the method of choice when the final application of biomass is food [10]. Solar dried mixture of corn and spirulina was successfully used in a fish feeding [10]. The sun drying is straightforward and cheap but weather-dependent and involves the possible risk of microbial contamination and spoilage, for longer drying periods, which in fact makes it less suitable for food and feed applications.

Drum drying

Drum dryers include a moving cylinder with facilities for deposition of the feed in thin layers on the hot surface of the drum and scraping blades to remove the dried layers. There are several designs of single, two or more drums which are widely used in different food, feed, and chemical applications. Drum drying was reported as the most common dehydration method in the 1980's [10]. Successful drum drying of *Scenedesmus* sp. was reported by Becker & Venkataraman [57]. In another study, drum and spray drying methods were compared for drying of microalgae [58]. In this study, 25% algae paste was used. The study suggested drum drying technique for drying of microalgae due to better results compared with spray drying, because of less investment, better digestibility of the dried biomass and lower energy requirement.

Cabinet drying

Cabinet drying can be categorised as direct air (cross flow) or vacuum cabinet drying [31, 58]. In a study conducted by Becker & Venkataraman [59], a biomass with 45% dry matter from *Spirulina* was dried in a cross flow cabinet dryer. The process was reported as cheaper than drum drying and more rapid than sun drying. As a benefit the cell wall of the microalgae was unbroken in contrast to the drum drying method [10]. Convective thin layer drying of microalgae *Spirulina* was studied by Desmorieux and Decaen [60]. In this study, sorption, isotherm of microalgae was measured and drying characterised by the drying kinetics. Effects of drying on the quality parameters were not reported.

Fluidised bed dryer

Fluid bed dryer has been successfully used in the food and pharmaceutical industries for the drying of particulate heat sensitive products. Leach et al. [61], investigated fluid bed drying of a high carotenoid *Dunaliella salina* biomass in calcium alginate beads with different alginate concentrations. Fluid bed dryer is not suitable for pastes and liquids, so normally it is not applied for the drying of microalgae. Drying of microalgae by spouted bed dryer was also reported in a recent study[62]. Compared to commonly used spray drying, The study revealed that spouted bed drying improved the lipid extractability by enhancement of cell rupture efficiency.

Spray drying

Spray drying is most likely the most favoured method of microalgae drying when the product is aimed for human consumption [9-10, 31]. The process is continuous and can handle large amounts of the biomass, while drying time is as low as few seconds. The algae are sprayed into the drying chamber trough a stream of hot gas. The dried product is removed afterwards from the bottom of the dryer. A cyclone, bag filter or both, recovers residual dust from the outgoing air stream [9]. This method is very efficient, but due to the high pressure induced by atomization process, microalgal cell wall rupture and subsequent loss of some bioactive compounds occur [31, 34, 59, 63]. Several studies have investigated the effect of the drying process on the cell structure, carotenoids and shelf life stability of several microalgae species [62- 67]. Generally speaking, spray-dried microalgae are more susceptible to oxidation than freeze-dried microalgae. Spray drying is the most expensive drying method excluding freeze-drying, and the spray dried algae powder is reportedly not as digestible as drum dried material [10, 34], which may make the method inappropriate for the production of fish feed ingredients.

Freeze drying

Freeze drying is a high quality, efficient and expensive drying technique, in which, the previously frozen sample, is being dehydrated under high vacuum. This method is typically applied to high-value products; however, freeze-drying is too expensive to be used for the large-scale commercial production of microalgal biomass [2, 9, 31,69]. Effects of freeze drying on the

quality and shelf life of microalgae biomass have previously been studied [64, 67- 70]. Compared to spray dried *Dunaliella salina*, more than 40% higher astaxanthin recovery in freeze-dried samples was reported by Ahmed et al. [69]. Guldhe et al. [70], investigated the effects of freeze drying, oven drying, and sun drying processes on lipid extraction yield and fatty acid composition of *Scenedesmus* sp. The highest contents of polyunsaturated fatty acids were observed in freeze-dried samples. Despite the excellent quality, freeze-dried microalgae biomass cannot be used as a fish meal replacer in the formulation of fish feed, as the processing expenses are very high. Freeze-drying is a non-destructive method by which, the cell structure and algae components remain unaffected. However, enzymes continued to be active during the storage of freeze dried microalgae, even at low temperatures, which resulted in unwanted degradations caused bye.g. lipolysis [68].

New trends- Pulse combustion spray drying

In a pulse combustion spray dryer, directions of drying gas and the substrate are opposite (counter current). Drying air accelerates to about 300 mph in a resonating pulse combustion engine, provides a high-velocity, powerful pulse wave, which atomises the droplets of sprayed liquid and drying happens in less than a second [55]. Some drying trials have been conducted in an algae production plant using this technology, but the results have not been reported yet. Table 2 summarizes different drying methods applied for microalgae as well as the most studied microalgae species, feed type and their advantages and disadvantages.

Selection of suitable downstream processing strategy

The downstream processing of microalgal biomass is known as a major and costly component of production. It normally includes a combination of two or more steps such as harvest, up concentration and drying, and is highly dependent on factors such as production volume and intended application of the final product(s). In **Figure. 1** different strategies for the production of dried biomass are shown. The selection of a proper strategy depends on several factors such as production capacity, the application of biomass (cosmetics, food/ feed, bioenergy/biofuel, etc.) and the local energy availability and costs.

Table 2. Different drying technologies applied to various microalgae species. N/A ; information is not available.

Drying method	Microalgae	Feed type	Advantages	Disadvantages	Reference
Solar drying	*Arthrospira* sp., *Scenedesmus* sp.	paste	Low cost, simple	Risk of microbial contamination, long drying time, product deterioration, variation in the quality of dried biomass, method is highly weather dependent	[10], [31], [59]
Rotary and drum drying	*Scenedesmus* sp.	Paste	Low cost, improves the digestibility of biomass as fish feed	Burning risk, low quality biomass due to high temperature, required high energy	[9], [34]
Cabinet and tunnel drying	*Arthrospira* sp., *Chlorella* sp. *Scenedesmus* sp.	Paste	Low cost, simple	Long drying time which may results in product deterioration, low efficiency, required more space	[10], [57], [62]
Fluidized & spouted bed drying	*Arthrospira* sp., *Dunaliela* sp.	Friable, granule, powder	High efficiency, rapid drying, good quality of dried biomass	Limitation in feed type requires actions such as back mixing.	[61], [62]
Spray drying	Various species such as; *Chlorella* sp., *Arthrospira* sp., *Nannochloropsis* sp., *Isocrysis* sp., *Haematococcus* sp.	Thixotropic, dilatant slurry	Destroys endogenous enzymes such as lipase, fast and efficient, produces homogenous powder	High capital and energy demands, lower digestibility of dried biomass, thermal decomposition of bioactive compounds such as carotenoids	[9], [10], [31], [34], [59], [63], [64], [65], [67]
Freeze drying	*Chlorella* sp., *Nannochloropsis* sp., *Dunaliela* sp., *Scenedesmus* sp.	Slurry, paste	High quality of dried biomass,	High energy consumption, suitable for small scale, Endogenous enzymes remain intact, lumpy biomass after drying which required milling	[2], [9], [63] [67], [68], [69], [70]

There is no single, globally accepted downstream processing method for microalgae. Most commercial facilities are developed for biofuel application so the quality of the product is not always as important as productivity. Spray drying of the harvested slurry (e.g. with 10-20 g/L DM) is the most common method for commercial production of algae powder. This process results in higher energy consumption as well as more deterioration of valuable compounds such as carotenoids, compared to freeze drying. However, when the microalgae are intended to be used as a fish feed ingredient, non-chemical dewatering consisting of microfiltration and centrifugation is suggestible. In this methodology, flocculants are not required which is a benefit, as some of these chemicals are not allowed to be used in the formulation of fish feed. On the other hand, the main part of the water can be separated in a physical, non-destructive manner without application of heat.

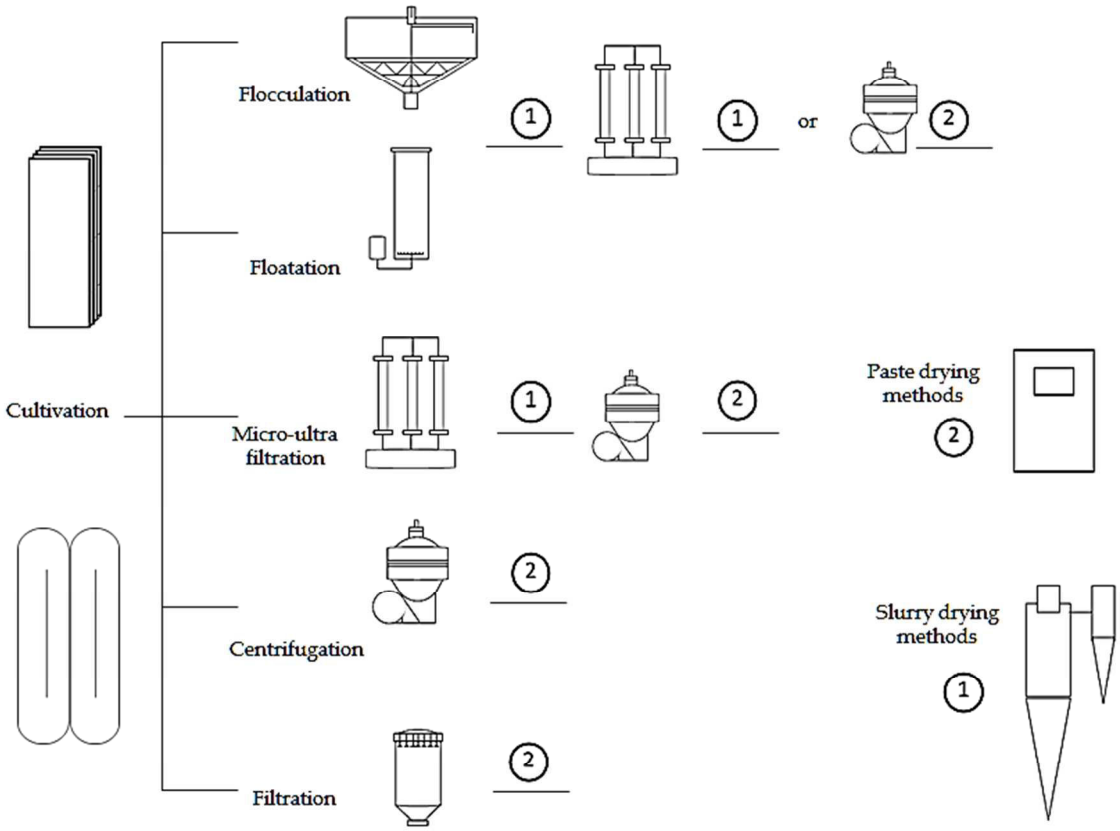

Figure 1. Different downstream processing strategies for the production of dried microalgae biomass.

Drying of the resulting paste (with 25-35% dry matter) requires less energy for the removing of excess water, compared to the slurry with 1-2 % dry matter. Drying of a high viscosity paste like this is not possible with spray drying.

It is necessary to use more delicate (albeit more expensive) drying technologies, such as freeze drying for high-value products. The production of fish feed ingredient with a comparable-reasonable price requires the development of new specific drying technologies or optimization of known method for feasible and gentle drying of microalgae paste.

Conclusion

When aiming at the utilisation of microalgae biomass as a feed ingredient the resulting biomass shall be produced in an economical approach while reasonably preserving valuable ingredients such as essential amino and fatty acids, pigments, and proteins. Harvest of microalgae by membrane microfiltration and subsequent up-concentration using an efficient and feasible centrifuge provides a biomass paste which includes nearly all of the bioactive compounds. Unfortunately, drying costs of freeze drying is very high, so the development of specific, feasible drying techniques wich can handle such high viscosity paste is critically required.

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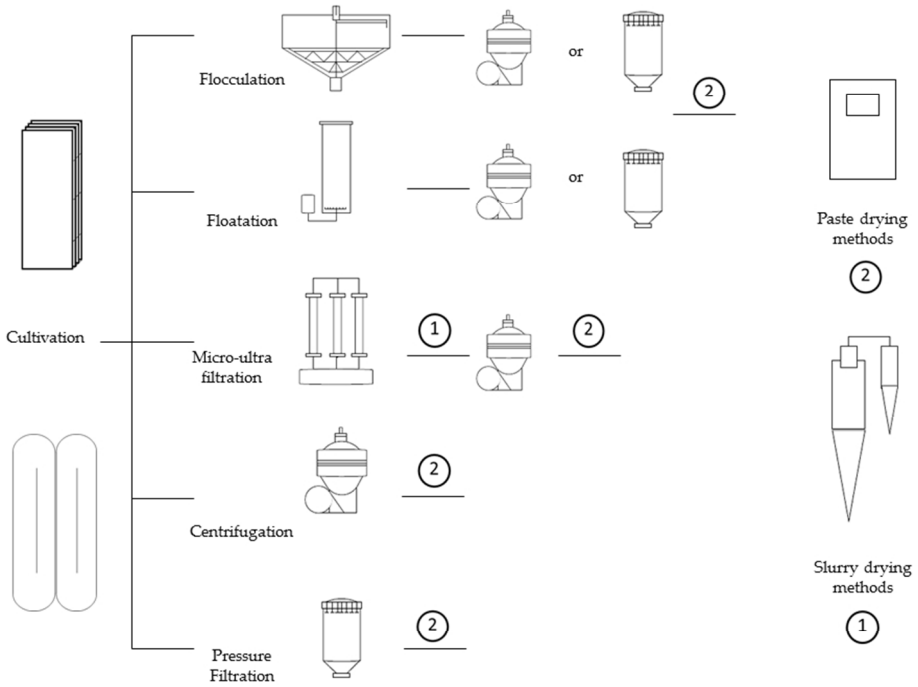
Harvest method	Microalgae	Recovery	Advantages	Disadvantages	Reference
Chemical & auto Flocculation	*Chlorella vulgaris, Choricystis minor, Cylindrotheca fusiformis, Neochloris* sp., *Nannochloropsis salina, Neochloris oleoabundans, Dunaniela salina, Chlorococcum* sp., *Chaetoceros calcitrans, Chaetoceros muelleri Chlorella, Isocrysis galbana, Pavlova lutheri, Tetraselmis* sp., *Thalassiosira pseudonana, Phaeodactylum tricornutum*	67-99 %	Simple, require low energy,	Requires chemicals (sometimes toxic), and subsequent separation techniques. Requires more space. Could not be used as a feed ingredient when non-feed grade chemicals are used.	[9], [14], [11],[17],[18],[22]
Bio- Flocculation	*Botryococcus braunii, Scenedesmus quadricauda, Selenastrum capricornutum, Anabaenaflos-aquae, Microcystis aeruginosa, Nannochloropsis oceanica, Pleurochrysis carterae, Chlorella* sp., *Pediastrum* sp., *Phormidium* sp., *Scenedesmus* sp., *consortium, Neochloris oleoabundans*	>83%	Non-toxic, low processing cost particularly for microalgae cultivated on waste water	Risk of contamination, variation in chemical composition, may require additional ingredients in the growth media	[11], [12], [16], [19], [20], [21], [23], [40], [22]
Ultrasonic assisted flocculation	*Monodus subterraneus, Euglena gracilis, Mychonastes aff. jurisii TKAC1031*	83 ± 12%	selective flocculation for some microalgae	Risk of cell rupture	[24], [25], [26]
Flotation	*Chlorella* sp. *Dunaliella salina, Tetraselmis* sp., *Microcystis aeruginosa, Asterionella formosa, Scenedesmus quadricauda, Melosira* sp.,	>85%	Requires low space , energy and processing time	Requires chemicals (sometimes toxic) and subsequent separation techniques. Not suitable for marine microalgae	[28], [11], [31], [10], [9], [30]
Electrical harvest methods	*Dunaliella salina, Chlorella* sp., *Microcystis aeruginosa,*	45-100 %	Efficient for fresh water algae	Requires relatively high energy. The emission of unwanted substances and H2 gas. Higher energy required for marine algae	[22], [12], [32]
Screen filtration, Pressure Filtration	*Arthruspira* sp., *Coelastrum proboscideum , Scenedesmus* sp., *Oscillatoria* sp.	< 90%	Simple, low maintenance and capital price	Suitable for very big microalgae cells, clogging,	[43], [12], [31]
Membrane filtration; Micro and ultra-filtration, reverse osmosis	*Chlorella* sp., *Phaeodactylum* sp., *Scenedesmus quadricauda , Nannochloropsis oculata, Nannochloropsis gaditana*	90-100%	Efficient, requires low energy. Low shear stress	clogging problems, High maintenance and operation costs for cleaning and changing of membranes, require higher pumping energy	[35], [36],[37], [38], [43], [69], [40], [44], [15], [42]
Centrifugations	*Scenedesmus* sp., *Coelastrum proboscideum Nannochloropsis* sp. ,*Chlorella* sp.,*Dunaliella* sp.,	90-100%	Fast, efficient, suitable for various cell sizes, even small microalgae such as *Nannochloropsis sp,*	High shear stress(disk stack), high capital and operation costs, not suitable for microalgae with thin cell walls	[10, [49], [12], [9], [48], [50], [51], [11], [31], [4]

Drying method	Microalgae	Feed type	Advantages	Disadvantages	Reference
Solar drying	*Arthrospira* sp., *Scenedesmus* sp.	paste	Low cost, simple	Risk of microbial contamination, long drying time, product deterioration, variation in the quality of dried biomass, method is highly weather dependent	[10], [31], [59]
Rotary and drum drying	*Scenedesmus* sp.	Paste	Low cost, improves the digestibility of biomass as fish feed	Burning risk, low quality biomass due to high temperature, required high energy	[9], [34]
Cabinet and tunnel drying	*Arthrospira* sp., *Chlorella* sp. *Scenedesmus* sp.	Paste	Low cost, simple	Long drying time which may results in product deterioration, low efficiency, required more space	[10], [57], [62]
Fluidized & spouted bed drying	*Arthrospira* sp., *Dunaliela* sp.	Friable, granule, powder	High efficiency, rapid drying, good quality of dried biomass	Limitation in feed type requires actions such as back mixing.	[61], [62]
Spray drying	Various species such as; *Chlorella* sp., *Arthrospira* sp., *Nannochloropsis* sp., *Isocrysis* sp., *Haematococcus* sp.	Thixotropic , dilatant slurry	Destroys endogenous enzymes such as lipase, fast and efficient, produces homogenous powder	High capital and energy demands, lower digestibility of dried biomass, thermal decomposition of bioactive compounds such as carotenoids	[9], [10], [31], [34], [59], [63], [64], [65], [67]
Freeze drying	*Chlorella sp.*,*Nannochloropsis* sp.,*Dunaliela* sp.,*Scenedesmus* sp.	Slurry, paste	High quality of dried biomass,	High energy consumption, suitable for small scale, Endogenous enzymes remain intact, lumpy biomass after drying which required milling	[2], [9], [63] [67], [68], [69], [70]



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Paper 6

Storage conditions affect oxidative stability and nutritional composition of 3 freeze-dried *Nannochloropsis salina.*

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Research Article

Storage conditions affect oxidative stability and nutritional composition of freeze-dried *Nannochloropsis salina*

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Running Title: Oxidation of dried *Nannochloropsis salina* during storage

Keywords: Oxidation, lipolysis, EPA, carotenoids, volatiles, acidity, peroxides

Abbreviations: Highly unsaturated fatty acids(HUFA); Eicosapentaenoic acid(EPA);Phospholipids(PL);Glycolipids(GL);Triacylglycerol(TAG), Long-chain polyunsaturated fatty acids(LC-PUFA), peroxide value (PV), free fatty acids (FFA)

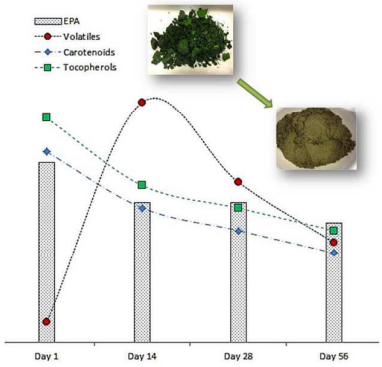
Abstract

Microalgae biomass is known as a promising sustainable source of bioactive compounds. Application of microalgae biomass in food and feed products requires information regarding storage stability and optimised storage conditions to minimise unwanted deterioration which downgrades the bioactive composition of microalgae biomass. In order to investigate the worsening of the nutritional quality of freeze dried biomass, a multifactorial storage experiment was conducted on a high EPA (eicosapentanoic acid) *Nannochloropsis salina* biomass. The storage time (0 to 56 days), storage temperature (5°C, 20°C and 40°C) and packaging conditions (under vacuum and ambient pressure) used as main factors. During the 56 days of storage, both time and temperature strongly influenced the oxidation reactions which result in deterioration of bioactive compounds such as carotenoids, tocopherols and EPA. Lipid deterioration occurred both due to enzyme-induced lipolysis and autoxidation. Carotenoids and α-tocopherol contents decreased during storage, but may still have prevented EPA from higher oxidative deteriorations due to their powerful antioxidant properties. Oxidation reactions, which resulted in the creation of primary and secondary products, occurred mainly at the first days of storage. The volatile compounds declined further probably due to reaction with amino acids, or decomposition to low molecular weight tertiary oxidation compounds. Storage of microalgae at low temperature is more effective than vacuum packaging.

Practical applications

Microalgae are known as a sustainable source of bioactive compounds, and their industrial scale application is growing very fast. Application of microalgae biomass in food, feed or cosmetics requires the knowledge of the optimum storage conditions to prevent the value-added compounds from deterioration. Results of this study improve our understanding of the chemical deterioration under different storage conditions and can help the producers/customers to extend the shelf life of microalgae biomass by choosing correct storage conditions.

Graphical abstract



The storage time and conditions influenced the bioactive compounds in freeze-dried microalgae biomass

1. Introduction

Microalgae are unicellular autotrophic organisms, which are found everywhere from the ice-covered Antarctic to hot and dry deserts. Microalgae are very diverse in size, morphology and cell composition. Depending on the species, growth stage and environmental factors, the main chemical composition in microalgae includes carbohydrates, lipids and proteins [1]. Microalgae also produce different valuable bioactive compounds such as vitamins, pigments and other natural antioxidants, which have specific and relevant applications [2]. Microalgal lipids accounts for 6-50% of dry cell weight [3], and include acylglycerols, phospholipids,

glycolipids, esters, sterols, tocopherols and hydrocarbons [4]. Lipids produced by microalgae can be categorised as storage and membrane lipids. Storage lipids consist of acylglycerols, which are mostly esterified with saturated and monounsaturated fatty acids such as palmitic acids and oleic acids. The majority of polyunsaturated fatty acids (PUFA) accumulate in the cell membrane in the form of polar lipids such as phospholipids and glycolipids [4, 5]. Studies have shown a higher bioavailability of n-3 fatty acids esterified to polar lipids such as phospholipids compared to the TAG esterified PUFA [6] . The thylakoid membranes consist of four glycerolipids of which monogalactosyldiacylglycerol and galactosyl diacylglycerol are dominant [7]. Highly unsaturated fatty acids (HUFA)play a major role in aquaculture nutrition. Marine fish typically require n-3 HUFA for optimal growth and health, which is primarily produced by the microalgae. Eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA) are considered as the essential fatty acids in the diet of aquatic animals [8, 9]. The content of EPA varies highly among the microalgae species. Eustigmatophytes such as *Nannochloropsis* sp., and diatoms such as *Phaeodactylum* sp. include a high percentage of EPA in their fatty acid composition [9, 10]. In green algae such as *Chlorella* sp. and *Dunaniella* and excluding rare species such as *Chlorella minutissima*, the percentage of C20 and C22 fatty acids in the fatty acid profile is very low, which make them unsuitable for single species aquatic diets[8].

Nannochloropsis salina is a promising microalga due to the high lipid (up to 50% DW) and EPA (up to 45% of total fatty acids) contents, when cultivated under proper conditions [10, 11]. The lipid fraction extracted from *Nannochloropsis salina* typically includes 8-10 % phospholipids (PL) and 30-40 % glycolipids (GL) and 50-60% triacylglycerol (TAG). The fatty acid composition includes C20:5 n-3, C16:0 and C16:1 n-9 as major fatty acids [10, 11]. While the high percentage of EPA in the microalgae biomass is an advantage from a nutritional point of view, this fatty acid is very sensitive to oxidation due to the high degree of unsaturation. Oxidation results in the decomposition of fatty acid and formation of primary (lipid hydroperoxides) and secondary oxidation products such as aldehydes and ketones. Furthermore, lipolysis caused by lipolytic enzymes in the microalgae may result in the

formation of free fatty acids. Both reactions will thus downgrade the nutritional quality of the biomass and reduce the storage stability of the biomass. The majority of the EPA in *Nannochloropsis salina* is primarily found in glycolipids and phospholipids. It was reported that EPA in the structure of polar lipid follows a different oxidation pathway, which is more complex compared to the TAG esterified HUFA's [12]. Lipid oxidation of FFA, PL and TAG will lead to the formation of undesirable off -flavours such as rancid and fishy, which reduce the acceptance of the lipids when used in both food and feed. On the other hand, it has been reported that autotrophic microalgae produce natural antioxidants to protect them from damage caused by oxidation [2]. The presence of various natural antioxidants such as tocopherols, carotenoids, and phenolics in microalgae biomass has also been reported in numerous studies [13].

Heat drying of microalgae biomass by methods such as spray drying results in the inactivation of the endogenous enzymes such as lipase and lipoxygenase, albeit at the expense of decomposition of the majority of valuable natural antioxidants such as carotenoids [14]. However, when using gentle drying methods such as freeze drying, natural compounds such as carotenoids and enzymes will remain nearly intact. During the wet and dry storage, shelf life stability of the microalgae biomass is being influenced by different factors such as natural antioxidant composition, packaging conditions and the storage time.

This study aimed at investigating the effects of storage time, temperature and packaging condition on the oxidative stability of a high EPA freeze-dried microalgae powder. The study also aimed to evaluate the variations of nutrients such as fatty acids, tocopherols and pigments of the microalgae biomass during the storage at various conditions.

2.Materials and methods

2.1. Chemicals and standards

Standards of fatty acids, volatiles and tocopherols were obtained from Sigma (St. Louise, USA) and Fluka (Deisenhofen, Germany). Standards of carotenoids were purchased from DHI (Hørsholm, Denmark). HPLC grade solvents were obtained from Sigma and Fluka. HPLC grade

water was prepared using a Milli-Q® Advantage A10 water deionizing unit from Millipore Corporation (Billerica, USA).

2.2. The microalgae biomass

The microalga *Nannohloropsis salina* was cultivated in flat panel photobioreactors under the conditions described by Safafar et al. [10]. The biomass was freeze dried immediately after harvest to a water content less than 10% in dry basis. The dried biomass was stored at -80°C.

2.3. Sample preparations and the experimental design

Microalgae biomass was hand ground gently and then mixed, packed and sealed in laminated aluminium bags (10cmx20cm) under reduced or atmospheric pressure with two replications. Packages stored at three different temperatures (5°C, 20°C and 40°C) for up to eight weeks. Data loggers monitored the variations in the storage temperature. Samples were analysed every 14 days from the start time.

2.4. Biochemical composition

Total lipids of all collected samples were extracted with chloroform/methanol according to the method described by Bligh&Dyer [15]. Analysis of tocopherols was done by HPLC-FLD according to an AOCS official method [16]. The fatty acid composition was determined by GLC-FID according to Safafar et al. [10]. Pigments were analysed by HPLC-DAD using the methods described by Safafar et al. [13].

2.5. Peroxide value

Peroxide value of the Bligh & Dyer extracted lipid fraction was determined according to the IDF official method [17]. The coloured complex of iron–thiocyanate was measured spectrometrically at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Kyoto, Japan). The results are expressed in milliequivalents peroxides per kg lipid extract (meq

O_2/kg lipid). The results were expressed as the absolute difference compared to the PV obtained at time 0.

2.6. Volatile compounds

The dynamic headspace procedure was used for the collection of the volatiles by using Tenax GR™ tubes. Around 0.5 g of sample (including 25 mg of 4-methyl-1-pentanol as internal standard) was mixed with 10 ml water and 1 ml antifoam and then purged under a flow of nitrogen (150 ml/min) in a water bath which was adjusted to 45 °C for 30 minutes. Afterwards, the dehydration of the Tenax tubes was done by a gentle flow of nitrogen (50 ml/min) for 20 minutes. Volatile compounds then were released from the Tenax matrix by using an automatic thermal desorbing unit (ATD-400, Perkin, Norwalk, USA). The autosampler head pressure was 9.2 psi, and the split rate was 5.0 mL/min. The desorption flow was 60 mL/min. Analysis of volatiles was performed by an Agilent 5890 II-A gas liquid chromatograph (Palo Alto, CA, USA) connected to an HP 5972 mass selective detector. The detector was set to 70 eV and measurements were done in the mass range between 30 and 350. Chromatographic separation of volatile compounds was performed on a DB1701 column (30 m × 0.25 mm × 0.5 µm film thickness) from J&W Scientific (Folsom, USA). Helium was used as a carrier gas under a constant flow of 1.3 mL/min. The temperature program was as follows: 3 min at 35 °C, from 35°C to 120 °C at the rate of 3 °C/min, from 120°C to 160 °C at the rate of 7/min, from 160 to200 °C at the rate of 15 °C/min and finally maintained at 200°C for 4 min. Quantification was done by calibration curves using a mixture of standards in pure ethanol. The standard mixture was put directly on the Tenax tubes and dehydrated subsequently for 5 min under a gentle flow of nitrogen. Calibration curve was made for each compound.

2.7. Free fatty acids (FFA)

Bligh& Dyer extract was used for the analysis of free fatty acid content. The extract (10 g) was mixed with 20 mL chloroform and 25 mL ethanol. The mixture was titrated in the presence of

phenolphthalein against 0.095 M NaOH ethanolic solution. The amount of free fatty acid content was calculated against the molar weight of EPA (302.45 g/mole) and result given in % FFA of total lipids.

2.9. Statistics

All experiments and analyses were performed in two replications except for analysis of volatile oxidation products which was performed in triplicate. Results are presented as mean ± std. Multiple factor ANOVA, with storage time (5 sampling points; 0-56 days), packaging condition (vacuum; VAC and ambient; AMB) and storage temperature (5, 20 and 40°C) as fixed factors were used to investigate the variations in the chemical composition and quality parameters of microalgae powder. Duncan's multiple range post-test was used to discriminate among the means at the 95% confidence level. All statistical procedures were performed in STATGRAPHICS software, version Centurion XVII (Stat point Technologies Inc., Warrenton, VA, USA).

3.Results and discussion;

3.1. Total lipids

The variations in the total lipid contents as a function of storage time, storage temperature and packaging conditions are shown in **Table 1**. According to the results of ANOVA, just storage time had statistically significant effect on the contents of lipids at the 95.0% confidence level (P-value <0.05). The packaging conditions and the interactions between factors were evaluated as not significant. These findings may be explained by the fact that the enzyme induced decompositions [2] may increase the extractability of the lipids at the first sampling time. In freeze-dried biomass, particles are flat and not spherical, so that these flat particles adhered together as layers [18]. This provides more contact between enzymes and their substrates. Besides this, the enzymes remain intact in freeze-dried microalgae biomass, compared to the spray dried biomass. Esquivel et al. [19] also reported such a decrease in lipid contents during dry storage of microalgae biomass.

3.2. Fatty acid compositions, Eicosapentaenoic acid(EPA)

Fatty acid composition in *Nannochloropsis salina* mostly includes C20 and C16 fatty acids, among them C20:5 n-3 (EPA) and C16:0 are the main fatty acids [10]. The variations in the EPA contents are shown in **Table 2.** Analysis of variances showed that only storage time and packaging condition had a significant effect on the EPA contents at 95% confidence level (P-value <0.05). After 56 days, EPA content in samples packed under vacuum was significantly higher compared to the ambient pressure-packed at 40°C, but not at lower temperatures. The lowest and highest loss in EPA contents after 56 days were observed for 5°C-VAC and 40°C-AMB experiments as -12.48 % and -15.87%, respectively. The EPA content declined faster during the first weeks of storage (between days 0 to 14) and then continued to decrease during the storage time, albeit at a lower rate. The effects of temperature on the stability of PUFA during the wet storage of microalgae biomass was previously reported by Welladsen et al. [20]. In that study, the highest decline was seen from 30 to 60 days storage in all experiments. There are various classes of natural antioxidants in the microalgae biomass, including tocopherols and carotenoids, which mostly remain intact during the freeze drying. These natural antioxidants might inhibit or retard lipid oxidation, which results in the EPA loss [2]. Hence, it cannot be ruled out that the presence of these antioxidants prevented a faster decomposition of EPA, particularly after two weeks of storage.

3.3. α-tocopherol

Like the other natural antioxidants tocopherols, which are known as free radical scavengers [21], accumulate in the microalgae cell to prevent the damages from external oxidative stresses such as photo-oxidation [2]. It has been shown previously that α-tocopherol is the major tocopherol in microalgae [13]. In *Nannochloropsis salina*, the amount of α-tocopherol in the biomass depends on several factors such as growth stage (harvest time), environmental conditions, and growth media composition [10]. During storage, the content of α-tocopherol was significantly influenced by the storage time and

packaging conditions at 95% confidence level (**Table 3**). Effects of temperature and the interactions among factors were evaluated as not significant. After 56 days storage, the lowest and highest losses were observed for 5°C-VAC and 40°C-AMB experiments as -14.57% and -67.92 %, respectively. These results suggested that α-tocopherol, as a natural antioxidant, might have contributed to decreasing the instability of EPA in microalgae biomass by scavenging of oxidation induced radicals, but in this process α-tocopherol decomposed. It is expected that EPA content will decline more drastically when the α-tocopherol has disappeared completely.

3.4. Carotenoids

Carotenoids including xanthophylls and carotenes have been demonstrated to be powerful natural antioxidants which are present in high concentrations in microalgae biomass [13, 20]. In *Nannochloropsis salina,* the carotenoid profile includes free and esterified vaucheriaxanthin, violaxanthin, canthaxanthin and β-carotene [10]. Carotenoids are thermolabile compounds, which decompose very fast during spray drying and other heating processes, while freeze-drying does not alter the pigment composition of microalgae biomass [14]. During storage, carotenes (β+α) decreased in all experiments (**Table 4**).

Analysis of variance demonstrated that the storage time, packaging conditions, and also interactions of time-temperature and time-temperature were as statistically significant factors at 95% confidence level (P value<0.05). The contents of carotenes drastically declined during the first 14 days of storage. At the end of storage time(day 56), the highest loss was measured in the samples stored at 40 °C (-94.08% for 40 °C – AMB). This loss was significantly higher compared to the samples stored at 20°C (-76%), and 5°C (- 69%). The effect of packaging conditions for the samples that were stored at 5 and 20°C was not statistically significant, whereas vacuum packing significantly reduced the decline in carotene content at 40 °C . Temperature sensitivity of carotenoids depends on their chemical structure. Carotenoids with a longer conjugated carbon-carbon double bond structure (e.g. β-carotene) are more heat-sensitive [14]. Based on these results it can be justified that that vacuum

packaging did not improve the stability of carotenes in the samples stored at lower temperatures, but only at high temperature.

Total xanthophylls (mainly include free and esterified vaucheriaxanthin, violaxanthin, canthaxanthin and minor compounds such as diadinoxanthin) also declined during storage as it shown in **Table 5**. Results of the multifactor statistical analysis revealed that the storage time, temperature and the interaction of time-temperature caused statistically significant changes in total xanthophylls. Effects of the packaging conditions and its corresponding interactions were evaluated as not significant ($p > 0.05$). Total xanthophylls declined in all samples during storage with the fastest decrease during the first 14 days. The decline during the first two weeks was larger at 40°C followed by 20°C and then 5°C. The highest loss during 56 days of storage was observed for the samples stored at 40°C under vacuum. It was somewhat surprising that vacuum did not reduce decomposition of xanthophylls. These findings are in agreement with the results obtained by Tang and Chen [21]. Similarly, Ryckebosch et al. [14] did not find any significant interaction between storage time and packaging conditions for carotenoid contents. Similar to the tocopherols, it has been suggested that carotenoids may protect the lipids from oxidising, but at the expense of their decomposition. Carotenoids with several conjugated double bonds are known as good singlet oxygen quenchers. The singlet oxygen quenching activity of carotenoids directly depends on the number of conjugated double bonds in their structure [21] so that β carotene and astaxanthin are potentially strong oxygen quenchers. Carotenoids can donate hydrogen to lipid peroxy radicals and produce stable carotene radicals due to the transfer of singlet electrons in its conjugated polyene. These radicals have enough lifetime to react with lipid peroxy radicals at low oxygen concentration (e.g. under vacuum) and generate non-radical carotene peroxides [22] which are not pigments anymore. These radicals can also transform to colourless carotene carbonyl and epoxides. A clear correlation between the disappearance of carotenoids and the inhibition of lipid peroxidation was also suggested previously [14]. In a previous study by Safafar et al. [10] it was shown that carotenoids, α-tocopherol and perhaps other natural antioxidants that were present in microalgae biomass prevented/retarded the

oxidative damages to the unsaturated fatty acids such as EPA during storage. It was shown that oxidation induced degradation of carotenoids results in an increase in short-chain mono and di-oxygenated compounds such as β-cyclocitral [23]. Similar results were reported by Sun et al. [24] ,who identified β-ionone and β-cyclocitral as main flavor compounds in the green macroalga *Capsosiphon fulvescens*.

3.5. Free fatty acids (FFA)

Enzyme-induced lipolysis has been suggested to be the biggest problem for the wet storage of microalgae. This activity results in the liberation of FFA, which are more sensitive to oxidation. The enzyme activity declines in the dried microalgal biomass [25]. Freeze-drying does not destroy the enzymes, and because the optimum activity temperature for lipase is 30-40°C, the storage temperature might influence the FFA contents in the microalgae biomass. The initial amounts of FFA in the biomass was high (17.2±1.20% total lipids). Compounds such as pigments and organic acids may interfere with the analysis, as a basic titration method was used in this study. To confirm that free fatty acids were as high as reported above, solid phase extraction of lipid classes of the extracted lipids, and subsequent quantitative determination of fatty acids were performed as described by Kim and Salem [26]. These results confirmed the findings observed by the titration method (data not shown). The high content of free fatty acids in freshly harvested microalgae has already been reported in several studies [27, 28]. Microalgae probably produce extracellular FFA, either to attract the bacteria or as a defence mechanism against the grazers, so the initial FFA contents of microalgae are high, especially for the algae harvested at autumn-winter [27]. It may also be attributed to the partial cell rupture caused by ice crystallisation, which occurs during the freezing stage of the freeze drying [29]. According to the results of ANOVA, both storage temperature and time significantly influenced the variations of FFA during the experiment. Effects of packaging conditions and the interactions between the factors were not statistically significant (**Figure 1**). However, higher contents of free fatty acids were observed in samples stored at 40°C and 20°C, compared to 5°C, as enzyme activity at 5°C is lower than at 20°C and 40°C.

3.6. Peroxide value (PV)

Lipid oxidation is a common stability problem in lipid-containing products. Oxidation of lipids can be initiated/accelerated by several factors such as heat, oxygen, light and pro-oxidants. The susceptibility of fatty acids to autoxidation depends on their relative ease to donate hydrogen [30]. For unsaturated fatty acids, susceptibility to oxidation directly depends on the availability of allylic hydrogens in the neighbourhood of double bonds for the reaction with radicals. Highly unsaturated fatty acids such as EPA oxidise very fast and produce primary oxidation products, which can be measured by peroxide value (PV), or level of conjugated dienes or trienes [2]. In this study, storage time and the interaction of temperature- packaging conditions significantly affected the absolute change in PV compared to time 0 (P-value <0.05). For samples stored at 40°C, PV increased until day 42, but then declined until last day of storage (**Figure 2**). The same trend was previously reported by Ryckebosch et al. [3]. The effects of storage temperature and packaging conditions on the variations of PV were not significant, while higher values were measured for the samples stored under vacuum and at 5°C. Hydroperoxides are unstable products, which decompose to several, mostly volatile compounds such as aldehydes and ketones. Hydroperoxide formation and decomposition involve a very complicated set of reaction pathways as shown in several studies [30]. On the other hand, pigments and individual carotenoids, which were present in microalgae biomass, may interfere in the absorbance spectra when colorimetric method like the one applied here are used. For this reason, the values reported here were obtained by deducting the observed PV from the PV at day 0. The same problem was declared for the measurement of PV in microalgae biomass [3]. It has been shown that free fatty acids are strong prooxidants [31, 32]. The prooxidant activity of the free fatty acids might be related to their ability to attract prooxidant metals and co-oxidise the esterified (e.g. triacylglycerol, glycolipids or phospholipids) fatty acids [32]. Evaluation of the correlation between PV and % free fatty acid at the 95.0% confidence level (P value <0.05), indicated a weak relationship

between these variables. A clearer and stronger correlation was probably dimmed due to the high standard deviations in the results of both FFA's and PV's.

3.7. Volatiles

Similar to what has been observed in plants, different volatile compounds may be produced by biological or chemical reactions in microalgae biomass [33]. These volatile compounds include hydrocarbons, terpenes, phenols, alcohols, aldehydes, ketones, esters and halogen or sulfur-containing compounds. The short chain linear aldehydes are often derived from chemical lipid oxidation while branched and aromatic aldehydes are typically formed due to enzymatic lipid and protein oxidation [23]. In addition to propanal, as a quantitatively important volatile deriving from autoxidation, 1-penten-3-one, 1-penten-3-ol, 2,4-heptadienal and pentadecane have been characterised as important volatile compounds contributing to the formation of off-flavor in LC-PUFA containing oils [34-36]. The variations in the selected volatile compounds including 1-penten-3-one, 1-penten-3-ol, 2-penten-1-ol, 2,4-heptadienal, 3-methyl butanal and pentadecane are shown in **Tables 6** and **7.**

Changes in 1-penten-3-one were mainly influenced by the storage time (P value <0.05). During the first days of storage, the amounts of 1-penten-3-one rapidly increased, reached a plateau at day 14 and then decreased, with the same pattern for samples stored at different temperatures. The highest amount was observed for the samples stored at 40°C at day 14, which was significantly greater than 5°C stored samples. Effect of packaging conditions was not significant, excluding the samples stored at 5°C (day 56).

Contents of 1-penten-3-ol, on the other hand, was significantly influenced by time, temperature, packaging conditions and the interactions of time-temperature (P value <0.05). The variation pattern was similar to 1-penten-3-one**,** with an incline between days 0 and 14, while the highest amounts were found in samples stored at 5°C, followed by 20°C and 40°C experiments, respectively.

It has been reported that 1-pentene-3-ol, which is derived from the oxidation pathway of EPA further oxidise onto 1-pentene-3-one [37]. The higher amounts of 1-penten-3-ol and

1-penten-3-one in samples stored under vacuum compared to ambient pressure were also reported by Benedetti and Mannino [34]. On the other hand, the presence of ketones such as 1-penten-3-one, 3-pentanone, and alcohols such as 1-penten-3-ol, in various strains of microalgae including *Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis* sp., *Nannochloropsis oculata*, and *Chlorella vulgaris* was reported by Van Durme et al. [23]. Considering the absence of EPA in the later species (*Chlorella vulgaris),* it can be concluded that other chemical or metabolic reactions may be involved in the production of these compounds and this could partly explain the high level of these compounds at time 0. Van Durme et al. [23] reported the contribution of 1- octen-3-ol to the formation of fish-like aroma.

Surprisingly, the variations in 3-methyl butanal content were influenced by the packaging conditions and also the interaction of time-temperature (P value <0.05). At the last day of storage, the highest contents of 3-methyl butanal were observed in the samples packed at ambient pressure and stored at 40°C, 20°C and 5°C respectively(P value >0.05). In contrast, significantly lower amounts of 3-methyl butanal were produced in the samples stored under vacuum. This compound is a strecker aldehyde formed from the reaction between aldehydes and amino acids present in the algae biomass.

The variability in the contents of 2-penten-1-ol was influenced by the storage time and the interaction of temperature and packaging conditions (P value <0.05). Again the same pattern as for 1-penten-3-one and 1-penten-3-one was found, with the highest amount at day 14, which subsequently declined to concentrations lower than that at day 0. Comparatively, the highest amounts were found in the samples stored at 5℃, followed by 20℃ and 40℃, respectively (P value >0.05).

The contents of 2,4-heptadienal were significantly influenced by the storage time, temperature and their interactions (P value <0.05), increasing very fast with a plateau at day 14 which then declined at a slower rate until day 56. Significantly higher amounts were found in the samples stored at 5°C, however, the difference between 20°C and 4°Cwas evaluated as not significant. 2, 4-heptadienal can further oxidise into pyrroles, which can happen faster in

40 °C. This may explain the higher contents of 2, 4-heptadienal in the samples stored at low temperature.

The pentadecane variations were not influenced by any of the fixed factors or their interactions (P value >0.05). However, comparatively higher amounts were detected in the samples stored at 40°C and ambient pressure. The pentadecane contents increased until day 42 and declined, albeit not significantly, until day 56.

Based on these results, oxidation of EPA and development of oxidation induced volatiles occured very fast and at the first days of storage and then decline through various reactions, such as decomposition to tertiary oxidation products. Volatiles can also have reacted with the amino acids in the microalgae biomass.

4.Conclusions;

During dry storage of microalgae *Nannochloropsis salina,* the storage time and temperature strongly influenced the oxidation reactions which results in deterioration of bioactive compounds such as carotenoids, tocopherols and LC PUFA and HUFA. In a freeze-dried microalgae biomass, the lipid deterioration occurred due to both enzyme-induced lipolysis and autoxidation. Carotenoids and α-tocopherol which are known as natural antioxidants, decomposed during the storage, while probably prevented/retarded oxidative deterioration of EPA. The oxidation reactions which resulted in the creation of primary and secondary products occurred mainly during the first days of storage. The volatile compounds declined further due to the formation of more stable compounds, e.g. by the bonding to some amino acids or decomposition of low molecular weight tertiary oxidation compounds. Storage of freeze-dried microalgae at a low temperature(e.g. 5°C) is more effective than oxygen-reduced storage conditions such as vacuum packaging. Further investigations are required to find additional methods for extending the shelf life of dried microalgae.

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Author Contributions:

All authors contributed to the writing and review of the manuscript and revising it critically for important intellectual content. All authors also contributed to final approval of the version to be submitted.

Conflicts of Interest:

The authors declare no conflict of interest.

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Table 1. Lipid content (% DW; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $P < 0.05$.

Table 2. Eicosapentanoic acid (EPA) contents (mg/g DW of the sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $P < 0.05$.

Table 3. α-Tocopherols contents (µg/g of the sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $p < 0.05$.

Table 4. Total carotene (µg/g of the sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $P < 0.05$.

Table 5. Total Xanthophylls (µg/g of DW sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q). $P < 0.05$.

Table 6. Contents of**; (a),**1-penten-3-ol **; (b),** 1-penten-3-one, and **(c),** 3-methyl-butanal (ng/g of DW sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q).$P < 0.05$.

Table 7. Contents of; **(a),** 2-penten-1-ol; **(b),** 2, 4 heptadienal, and **(c)**, pentadecane (ng/g of DW sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q). $P < 0.05$.

Figure 1. Free fatty acid contents (% of lipids DW; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. The values presented here were deducted from the free fatty acid content at day 0, (17.2±1.20% total lipids). For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q). Bars are representing the standard deviations, $P < 0.05$.

Figure 2. Absolute changes in peroxide values compared with day 0 (Meq g O2 /g lipids DW; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. The values reported here were obtained by deducting the observed PV from the PV at day 0. Bars are representing the standard deviations.

Tables;

Table 1.

	5°C		20°C		40°C	
Time(day)	VAC	AMB	VAC	AMB	VAC	AMB
0	22.2±0.34 ab,x, p	22.2±0.34 b,x,p	22.2±0.34 a,x,p	22.2±0.34 b,x,p	22.2±0.34 b,x,p	22.2±0.30 a,x,p
14	22.5±0.06 b,x, p	21.9±0.05 ab,x,p	25.2±0.82 c,y,q	24.1±0.43 c,y,p	25.4±0.65 c,y,p	25.3±0.61 c,y,p
28	21.1±0.15 a,x,p	22.1±0.24 a,y,q	22.5±2.30 b,y,q	20.6±1.95 a,x,p	23.0±1.87 b,z,p	22.9±0.24 b,y,p
42	21.2±0.58 a,x,p	20.9±1.07 ax,p	22.1±1.03 a,y, q	21.2±0.14 a,x,p	22.1±1.31 a,y,p	22.1±0.81 a,y,p
56	21.4±0.11 a,x, p	21.4±0.72 a,x,p	21.3±0.51 a,x,p	22.8±1.77 b, y, q	21.4±0.91 a,x, p	21.3±0.61 a,x,p

Table 2.

	5°C		20°C		40°C	
Time(day)	VAC	AMB	VAC	AMB	VAC	AMB
0	94.5±2.32 b,w,p	94.5±2.32 d,w,p	94.5±2.32 c,w,p	94.5±2.32 c,w,p	94.5±2.32 c,w,p	94.5±2.32 c,w,p
14	92.4±0.43 b,x,p	90.8±1.2 c, x,p	91.4±1.41 bc,x,q	85.9±2.72 b,w,p	91.9±4.51 bc,x,q	86.6±2.21 b, w,p
28	85.0±5.17 b,x,p	86.9±0.59 b,x,p	88.5±5.22 b,y,q	76.1±3.14 a,w,p	89.2±0.94 b,y,q	86.8±0.22 b,x,p
42	81.7±7.09 a,w,p	83.2±2.8 a,x,p	84.2±2.86 a,x,q	78.6±2.14 a,w,p	82.3±2.04 a,x,p	81.8±2.19 a,w,p
56	82.7±4.57 a,x,p	81.8±0.23 a,w,p	83.0±3.24 a,x,p	81.0±1.63 a,w,p	83.3±1.37 a,x,q	79.5±1.56 a,w,p

Table 3.

	5°C		20°C		40°C	
Time(day)	VAC	AMB	VAC	AMB	VAC	AMB
0	460.5±67.1 bc,w,p	460.5±67.1 c,w,p	460.5±67.1 b,w,p	460.5±67.1 c,w,p	460.5±67.1 b,w,p	460.5±67.1 c,w,p
14	411.7±63.3 b,x,q	310.2±113.6 a,w,p	452.9±15.8 b,x,q	386.5±29.9 b,wx,p	305.8±90.2 a,w,p	302.2±15.3 b,w,p
28	308.6±61.6 a,wx,q	264.2±7.67 a,w,p	525.1±94.1 bc,z,q	342.2±22. 3 ab, x,p	494.8±86.1 bc,y,q	291.1±49.1 b,w,p
42	415.2±82.8 b,x,p	409.8±4.81 bc,x,p	434.75±11.2 b,x,q	316.39±12.7 a,w,p	437.5±44.1 b,x,q	265.1±49.3 b,w,p
56	393.4±56.2 ab,y,p	351.6±12.5 ab,xy,p	362.76±8.25 a,xy,p	297.98±34.5 a,x,p	313.11±9.24 a,x,q	147.7±43.9 a,w,p

Table 4.

Temperature(°C)	Packing	Storage time (day) 0	14	28	42	56
5	VAC	2418± 16.7 d,w,p	951.1± 22.3 b,xy,q	710.4± 57.9 a,w,p	1137± 85.82 c,y,q	747.4±24.6 a,y,p
	AMB	2418± 16.7 c,w,p	895.5± 66.5 b,x,p	647.6± 51.9 a,w,p	623.4± 93.43 a,w,p	646.1± 38.1 a,x,p
20	VAC	2418± 16.7 d,w,p	629.7±76.7 a,w,p	906.1±105.3 c,y,q	715.1± 27.17 b, x,q	571.3± 90.8 a,x,p
	AMB	2418± 16.7 c,w,p	608.4±177.7 a,w,p	705.8±34.6 b,w,p	596.5±71.23 a,w,p	790.8±150. 1 b,y,q
40	VAC	2418±16.7 d,w,p	875.1± 78.4 bc,x,p	738.3±90.9 b,w,p	1032±262.1 c,y,q	220.4±74.0 a,w,q
	AMB	2418±16.7 d,w,p	854.3± 175.4 bc,x,p	771.5± 82.0 c,wx,p	577.8±158.1 b,w,p	143.1±42.5 a,w,p

Table 5.

Temperature(°C)	Packing	Storage time (day) 0	14	28	42	56
5	VAC	17861±134.1 b,w,p	9563±189.7 a,y,q	9491±627.1 a,y,p	8820± 182.8 a,z,p	9062±726.1 a,z,p
	AMB	17861±134.1 b,w,p	9318±783.8 a,y,p	9370± 298.8 a,y,p	8749±209.9 a,z,p	9189±1389 a,z,p
20	VAC	17861±134.1 e,w,p	8998±113.5 d,xy,q	6842±843.2 c,x,p	2594±96.3 a,w,p	3923±281.3 b,x,p
	AMB	17861±134.1 e,w,p	8107±195.4 c,x,p	6624±87.0 b,x,p	4369±2905 a,y,q	6442±341.7 b,y,q
40	VAC	17861±134.1 e,w,p	4062±27.7 bc,w,p	3811±108.3 b,w,p	4974±373.3 c,y,q	874.5±98.4 a,w,p
	AMB	17861±134.1 e,w,p	4497±278.1 c,w,p	3749± 635.7 b,w,p	3393±111.8 b,x,p	1100±440.3 a,w,p

Table 6.

1-penten-3-ol

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	3992±342,3	b,x	3992±342,3	a,x	3992±342,3	a,x
	AMB	8479±567,6	d,x,p	7214±1179	b,x,p	10593±357,2	d,y,p
14	VAC	8162±595,5	d,x,p	12699±1317	d,y,q	11619±3217	d,y,p
	AMB	4479±655,5	bc,x,p	4845±648,8	a,x,p	9715±1294	c,y,p
28	VAC	4526±1018	bc,x,p	9426±3747	c,y,q	10523±338,2	d,y,q
	AMB	5527±271,8	c,x,p	8958±203,6	bc,y,q	4118±507,7	ab,x,p
42	VAC	8892±1910	d,y,q	3187±196,1	a,x,p	9280±1299	c,y,q
	AMB	3570±445,5	a,x,q	3023±64,05	a,x,p	5415±1268	b,y,p
56	VAC	2053±433,3	a,x,p	4135±248,9	a,xy,p	5133±1251	b,y,p

(a)

1-penten-3-one

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	1902±238.2	a,x	1902±238.2	a,x	1902±238.2	a,x
	AMB	8413±312.5	e,z,p	5450±1319	d,y,p	3798±383.1	c,x,q
14	VAC	8368±773.9	e,z,p	4888±900.7	c,y,p	2370±1707	a,x,p
	AMB	4013±1008	c,x,p	4465±891.9	c,x,p	3932±2100	c,x,q
28	VAC	5463±1881	d,y,q	4793±1500	c,y,p	2173±251.8	a,x,p
	AMB	5172±583.9	d,y,q	2707±980.9	a,x,p	6223±1623	d,z,q
42	VAC	4496±1100	c,y,p	2687±349.4	b,x,p	3383±646.5	b,y,p
	AMB	3232±448.8	b,x,p	2848±250.5	b,x,p	3082±969.1	b,xq
56	VAC	2764±782.1	a,y,p	2633±81.16	a,xy,p	2000±505.3	a,x,p

(b)

3-methyl-butanal

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	940.1±77	b,x	940.1±77	b,x	940.1±77	b,x
	AMB	2176±98.43	d,z,q	1725±265.1	d,y,q	1056±73.44	bc,x,p
14	VAC	1067±93.72	b,x,p	987.9±89.08	bc,x,p	1020±189	bc,x,p
	AMB	2176±416.1	d,y,q	1036±66.20	c,x,q	873.9±149.9	ab,x,p
28	VAC	853.1±57.61	ab,y,p	489.4±186.9	a,x,p	1120±26.23	c,z,q
	AMB	1181±34.2	b,xy,q	863.5±106.5	b,x,p	715.8±284.7	a,x,q
42	VAC	742.5±40.46	a,x,p	1583±109.7	d,z,q	1009±136.3	bc,y,p
	AMB	1736±199.5	c,z,q	1036±35.30	c,y,q	643.3±132.1	a,x,p
56	VAC	560.3±26.33	a,x,p	631.3±75.63	a,x,p	585.6±89.58	a,x,p

(c)

Table 7.

2-penten-1-ol

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	3044±787.3	bc,x	3044±787.3	bc,x	3044±787.3	c,x
14	AMB	4730±876.1	cd,y,p	3537±643.7	c,x,p	4272±331.3	c,x,p
	VAC	4870±513.5	d,xy,p	5585±639.2	d,y,q	4543±1795	cd,x,p
28	AMB	1530±168.7	ab,x,p	2159±359.9	b,xy,p	4719±426.6	d,y,p
	VAC	2725±755.4	b,x,q	4123±1806	c,y,q	4415±307.9	cd,y,p
42	AMB	2475±244.3	b,y,p	570.7±47.17	a,x,p	1370±134.0	a,x,p
	VAC	3894±829.5	c,y,q	820.7±69.85	a,x,p	3972±562.6	c,y,q
56	AMB	879.9±173.4	a,x,p	1156±114.2	a,x,p	2442±581.8	b,y,p
	VAC	965.8±300.8	a,x,p	2063±319.6	b,y,q	2124±542.1	ab,y,p

(a)

2,4 heptadienal

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	119.4±22.00	a,x	119.4±22.00	a,x	119.4±22.00	a,x
14	AMB	588.5±43.69	e,z,p	384.6±75.17	cd,x,p	483.3±38.33	c,y,p
	VAC	524.6±47.60	de,y,p	524.3±28.78	e,y,q	463.8±156.0	c,x,p
28	AMB	364.2±30.29	c,y,p	420.5±50.94	d,y,p	612.1±57.53	e,z,q
	VAC	369.1±128.6	c,x,p	441.4±128.0	d,y,p	457.6±37.46	c,y,p
42	AMB	483.0±17.49	d,y,q	443.4±66.48	d,y,q	381.1±82.51	b,x,p
	VAC	379.8±45.74	c,x,p	358.2±28.89	b,x,p	558.5±82.89	d,y,q
56	AMB	387.4±15.96	c,x,q	355.7±58.20	b,x,p	428.2±60.81	c,y,q
	VAC	276.1±82.45	b,x,p	314.7±21.45	b,y,p	338.8±34.66	b,y,p

(b)

pentadecane

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	417.0±112.1	a,x	417.0±112.1	a,x	417.0±112.1	a,x
14	AMB	1456±321.4	b,x,p	984.0±222.9	b,x,p	889.1±125.9	ab,x,p
	VAC	1018±117.4	b,x,p	1025±349.9	b,x,p	503.8±316.3	a,x,p
28	AMB	201.0±40.08	a,x,p	512.0±80.47	a,x,p	809.5±81.47	ab,x,p
	VAC	537.4±135.9	a,x,p	349.8±54.84	a,x,p	836.3±118.2	ab,x,p
42	AMB	527.8±72.38	a,x,p	679.0±18.83	a,x,p	1205±175.7	b,x,p
	VAC	725.9±103.6	a,x,p	123.5±28.85	a,x,p	769.3±23.63	a,x,p
56	AMB	124.2±20.40	a,x,p	245.8±16.96	a,x,p	685.5±77.67	a,x,p
	VAC	429.6±34.45	a,x,p	453.0±112.7	a,x,p	323.9±72.58	a,x,p

(C)

Figures;

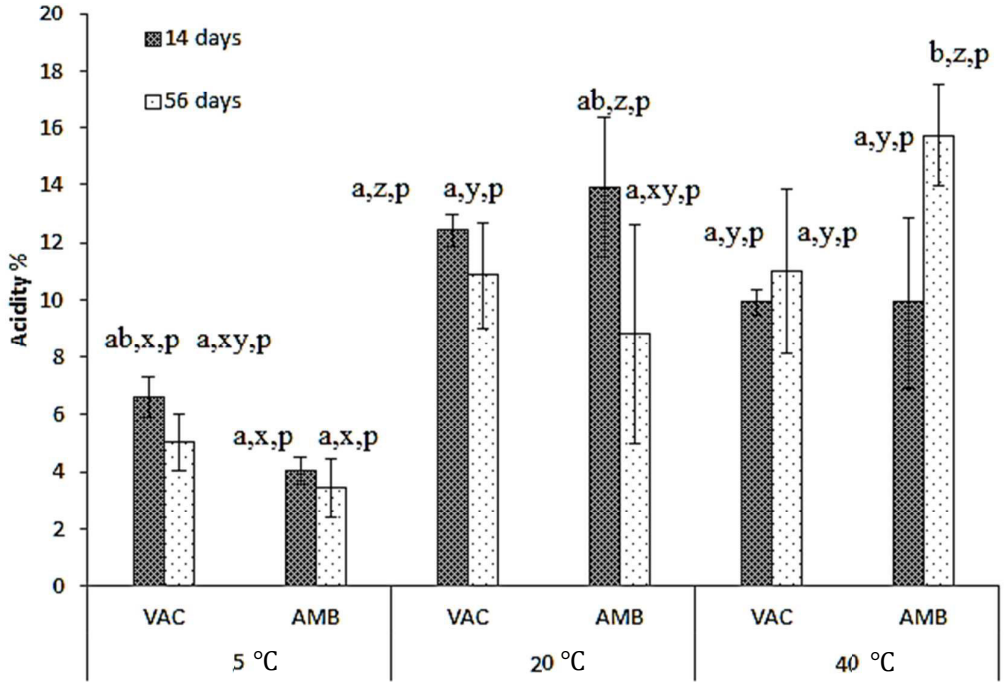

Figure 1.

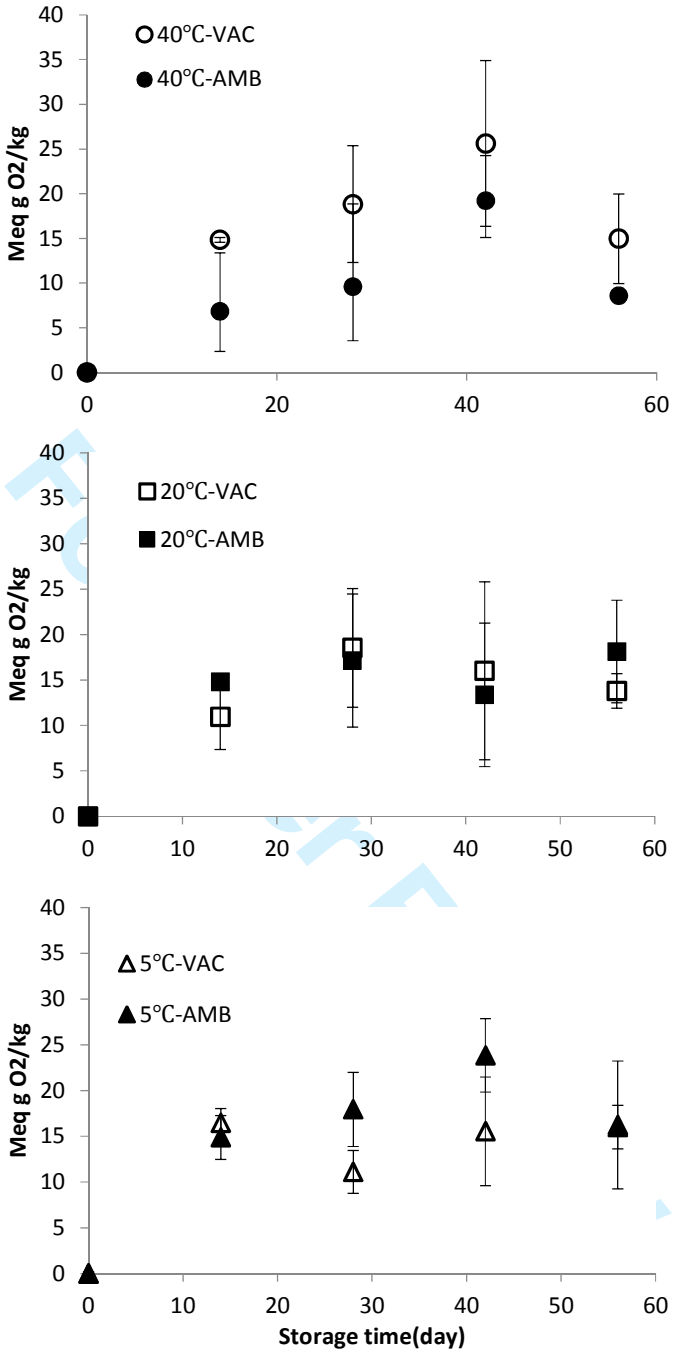
40°C-VAC
40°C-AMB
20°C-VAC
20°C-AMB
5°C-VAC
5°C-AMB
Meq g O2/kg
Storage time(day)

Figure 2.

Tables;

Table 1. Lipid content (% DW; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $P < 0.05$.

	5°C		20°C		40°C	
Time(day)	VAC	AMB	VAC	AMB	VAC	AMB
0	22.2±0.34 ab,x, p	22.2±0.34 b,x,p	22.2±0.34 a,x,p	22.2±0.34 b,x,p	22.2±0.34 b,x,p	22.2±0.30 a,x,p
14	22.5±0.06 b,x, p	21.9±0.05 ab,x,p	25.2±0.82 c,y,q	24.1±0.43 c,y,p	25.4±0.65 c,y,p	25.3±0.61 c,y,p
28	21.1±0.15 a,x,p	22.1±0.24 a,y,q	22.5±2.30 b,y,q	20.6±1.95 a,x,p	23.0±1.87 b,z,p	22.9±0.24 b,y,p
42	21.2±0.58 a,x,p	20.9±1.07 ax,p	22.1±1.03 a,y, q	21.2±0.14 a,x,p	22.1±1.31 a,y,p	22.1±0.81 a,y,p
56	21.4±0.11 a,x, p	21.4±0.72 a,x,p	21.3±0.51 a,x,p	22.8±1.77 b, y, q	21.4±0.91 a,x, p	21.3±0.61 a,x,p

Table 2. Eicosapentanoic acid (EPA) contents (mg/g DW of the sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $P < 0.05$.

	5°C		20°C		40°C	
Time(day)	VAC	AMB	VAC	AMB	VAC	AMB
0	94.5±2.32 b,w,p	94.5±2.32 d,w,p	94.5±2.32 c,w,p	94.5±2.32 c,w,p	94.5±2.32 c,w,p	94.5±2.32 c,w,p
14	92.4±0.43 b,x,p	90.8±1.2 c, x,p	91.4±1.41 bc,x,q	85.9±2.72 b,w,p	91.9±4.51 bc,x,q	86.6±2.21 b, w,p
28	85.0±5.17 b,x,p	86.9±0.59 b,x,p	88.5±5.22 b,y,q	76.1±3.14 a,w,p	89.2±0.94 b,y,q	86.8±0.22 b,x,p
42	81.7±7.09 a,w,p	83.2±2.8 a,x,p	84.2±2.86 a,x,q	78.6±2.14 a,w,p	82.3±2.04 a,x,p	81.8±2.19 a,w,p
56	82.7±4.57 a,x,p	81.8±0.23 a,w,p	83.0±3.24 a,x,p	81.0±1.63 a,w,p	83.3±1.37 a,x,q	79.5±1.56 a,w,p

Table 3. α-Tocopherols contents (μg/g of the sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $p < 0.05$.

	5°C		20°C		40°C	
Time(day)	VAC	AMB	VAC	AMB	VAC	AMB
0	460.5±67.1 bc,w,p	460.5±67.1 c,w,p	460.5±67.1 b,w,p	460.5±67.1 c,w,p	460.5±67.1 b,w,p	460.5±67.1 c,w,p
14	411.7±63.3 b,x,q	310.2±113.6 a,w,p	452.9±15.8 b,x,q	386.5±29.9 b,wx,p	305.8±90.2 a,w,p	302.2±15.3 b,w,p
28	308.6±61.6 a,wx,q	264.2±7.67 a,w,p	525.1±94.1 bc,z,q	342.2±22. 3 ab, x,p	494.8±86.1 bc,y,q	291.1±49.1 b,w,p
42	415.2±82.8 b,x,p	409.8±4.81 bc,x,p	434.75±11.2 b,x,q	316.39±12.7 a,w,p	437.5±44.1 b,x,q	265.1±49.3 b,w,p
56	393.4±56.2 ab,y,p	351.6±12.5 ab,xy,p	362.76±8.25 a,xy,p	297.98±34.5 a,x,p	313.11±9.24 a,x,q	147.7±43.9 a,w,p

Table 4. Total carotene (μg/g of the sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging condition (p-q). $P < 0.05$.

		Storage time (day)				
Temperature(°C)	Packing	0	14	28	42	56
5	VAC	2418± 16.7 d,w,p	951.1± 22.3 b,xy,q	710.4± 57.9 a,w,p	1137± 85.82 c,y,q	747.4±24.6 a,y,p
	AMB	2418± 16.7 c,w,p	895.5± 66.5 b,x,p	647.6± 51.9 a,w,p	623.4± 93.43 a,w,p	646.1± 38.1 a,x,p
20	VAC	2418± 16.7 d,w,p	629.7±76.7 a,w,p	906.1±105.3 c,y,q	715.1± 27.17 b, x,q	571.3± 90.8 a,x,p
	AMB	2418± 16.7 c,w,p	608.4±177.7 a,w,p	705.8±34.6 b,w,p	596.5±71.23 a,w,p	790.8±150. 1 b,y,q
40	VAC	2418±16.7 d,w,p	875.1± 78.4 bc,x,p	738.3±90.9 b,w,p	1032±262.1 c,y,q	220.4±74.0 a,w,q
	AMB	2418±16.7 d,w,p	854.3± 175.4 bc,x,p	771.5± 82.0 c,wx,p	577.8±158.1 b,w,p	143.1±42.5 a,w,p

Table 5. Total Xanthophylls (μg/g of DW sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q). $P < 0.05$.

		Storage time (day)				
Temperature(°C)	Packing	0	14	28	42	56
5	VAC	17861±134.1 b,w,p	9563±189.7 a,y,q	9491±627.1 a,y,p	8820± 182.8 a,z,p	9062±726.1 a,z,p
	AMB	17861±134.1 b,w,p	9318±783.8 a,y,p	9370± 298.8 a,y,p	8749±209.9 a,z,p	9189±1389 a,z,p
20	VAC	17861±134.1 e,w,p	8998±113.5 d,xy,q	6842±843.2 c,x,p	2594±96.3 a,w,p	3923±281.3 b,x,p
	AMB	17861±134.1 e,w,p	8107±195.4 c,x,p	6624±87.0 b,x,p	4369±2905 a,y,q	6442±341.7 b,y,q
40	VAC	17861±134.1 e,w,p	4062±27.7 bc,w,p	3811±108.3 b,w,p	4974±373.3 c,y,q	874.5±98.4 a,w,p
	AMB	17861±134.1 e,w,p	4497±278.1 c,w,p	3749± 635.7 b,w,p	3393±111.8 b,x,p	1100±440.3 a,w,p

Table 6. Contents of**; (a),**1-penten-3-ol **; (b),** 1-penten-3-one, and **(c),** 3-methyl-butanal (ng/g of DW sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q).P < 0.05.

1-penten-3-ol

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	3992±342,3	b,x	3992±342,3	a,x	3992±342,3	a,x
14	AMB	8479±567,6	d,x,p	7214±1179	b,x,p	10593±357,2	d,y,p
	VAC	8162±595,5	d,x,p	12699±1317	d,y,q	11619±3217	d,y,p
28	AMB	4479±655,5	bc,x,p	4845±648,8	a,x,p	9715±1294	c,y,p
	VAC	4526±1018	bc,x,p	9426±3747	c,y,q	10523±338,2	d,y,q
42	AMB	5527±271,8	c,x,p	8958±203,6	bc,y,q	4118±507,7	ab,x,p
	VAC	8892±1910	d,y,q	3187±196,1	a,x,p	9280±1299	c,y,q
56	AMB	3570±445,5	a,x,q	3023±64,05	a,x,p	5415±1268	b,y,p
	VAC	2053±433,3	a,x,p	4135±248,9	a,xy,p	5133±1251	b,y,p

(a)

1-penten-3-one

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	1902±238.2	a,x	1902±238.2	a,x	1902±238.2	a,x
14	AMB	8413±312.5	e,z,p	5450±1319	d,y,p	3798±383.1	c,x,q
	VAC	8368±773.9	e,z,p	4888±900.7	c,y,p	2370±1707	a,x,p
28	AMB	4013±1008	c,x,p	4465±891.9	c,x,p	3932±2100	c,x,q
	VAC	5463±1881	d,y,q	4793±1500	c,y,p	2173±251.8	a,x,p
42	AMB	5172±583.9	d,y,q	2707±980.9	a,x,p	6223±1623	d,z,q
	VAC	4496±1100	c,y,p	2687±349.4	b,x,p	3383±646.5	b,y,p
56	AMB	3232±448.8	b,x,p	2848±250.5	b,x,p	3082±969.1	b,xq
	VAC	2764±782.1	a,y,p	2633±81.16	a,xy,p	2000±505.3	a,x,p

(b)

3-methyl-butanal

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	940.1±77	b,x	940.1±77	b,x	940.1±77	b,x
14	AMB	2176±98.43	d,z,q	1725±265.1	d,y,q	1056±73.44	bc,x,p
	VAC	1067±93.72	b,x,p	987.9±89.08	bc,x,p	1020±189	bc,x,p
28	AMB	2176±416.1	d,y,q	1036±66.20	c,x,q	873.9±149.9	ab,x,p
	VAC	853.1±57.61	ab,y,p	489.4±186.9	a,x,p	1120±26.23	c,z,q
42	AMB	1181±34.2	b,xy,q	863.5±106.5	b,x,p	715.8±284.7	a,x,q
	VAC	742.5±40.46	a,x,p	1583±109.7	d,z,q	1009±136.3	bc,y,p
56	AMB	1736±199.5	c,z,q	1036±35.30	c,y,q	643.3±132.1	a,x,p
	VAC	560.3±26.33	a,x,p	631.3±75.63	a,x,p	585.6±89.58	a,x,p

(c)

Table 7. Contents of; **(a),** 2-penten-1-ol; **(b),** 2, 4 heptadienal, and **(c)**, pentadecane (ng/g of DW sample; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q). $P < 0.05$.

2-penten-1-ol

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	3044±787.3	bc,x	3044±787.3	bc,x	3044±787.3	c,x
14	AMB	4730±876.1	cd,y,p	3537±643.7	c,x,p	4272±331.3	c,x,p
	VAC	4870±513.5	d,xy,p	5585±639.2	d,y,q	4543±1795	cd,x,p
28	AMB	1530±168.7	ab,x,p	2159±359.9	b,xy,p	4719±426.6	d,y,p
	VAC	2725±755.4	b,x,q	4123±1806	c,y,q	4415±307.9	cd,y,p
42	AMB	2475±244.3	b,y,p	570.7±47.17	a,x,p	1370±134.0	a,x,p
	VAC	3894±829.5	c,y,q	820.7±69.85	a,x,p	3972±562.6	c,y,q
56	AMB	879.9±173.4	a,x,p	1156±114.2	a,x,p	2442±581.8	b,y,p
	VAC	965.8±300.8	a,x,p	2063±319.6	b,y,q	2124±542.1	ab,y,p

(a)

2,4 heptadienal

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	119.4±22.00	a,x	119.4±22.00	a,x	119.4±22.00	a,x
14	AMB	588.5±43.69	e,z,p	384.6±75.17	cd,x,p	483.3±38.33	c,y,p
	VAC	524.6±47.60	de,y,p	524.3±28.78	e,y,q	463.8±156.0	c,x,p
28	AMB	364.2±30.29	c,y,p	420.5±50.94	d,y,p	612.1±57.53	e,z,q
	VAC	369.1±128.6	c,x,p	441.4±128.0	d,y,p	457.6±37.46	c,y,p
42	AMB	483.0±17.49	d,y,q	443.4±66.48	d,y,q	381.1±82.51	b,x,p
	VAC	379.8±45.74	c,x,p	358.2±28.89	b,x,p	558.5±82.89	d,y,q
56	AMB	387.4±15.96	c,x,q	355.7±58.20	b,x,p	428.2±60.81	c,y,q
	VAC	276.1±82.45	b,x,p	314.7±21.45	b,y,p	338.8±34.66	b,y,p

(b)

pentadecane

Storage time(day)		Temperature (°C)					
	Pack	40		20		5	
0	-	417.0±112.1	a,x	417.0±112.1	a,x	417.0±112.1	a,x
14	AMB	1456±321.4	b,x,p	984.0±222.9	b,x,p	889.1±125.9	ab,x,p
	VAC	1018±117.4	b,x,p	1025±349.9	b,x,p	503.8±316.3	a,x,p
28	AMB	201.0±40.08	a,x,p	512.0±80.47	a,x,p	809.5±81.47	ab,x,p
	VAC	537.4±135.9	a,x,p	349.8±54.84	a,x,p	836.3±118.2	ab,x,p
42	AMB	527.8±72.38	a,x,p	679.0±18.83	a,x,p	1205±175.7	b,x,p
	VAC	725.9±103.6	a,x,p	123.5±28.85	a,x,p	769.3±23.63	a,x,p
56	AMB	124.2±20.40	a,x,p	245.8±16.96	a,x,p	685.5±77.67	a,x,p
	VAC	429.6±34.45	a,x,p	453.0±112.7	a,x,p	323.9±72.58	a,x,p

(C)

Figures;

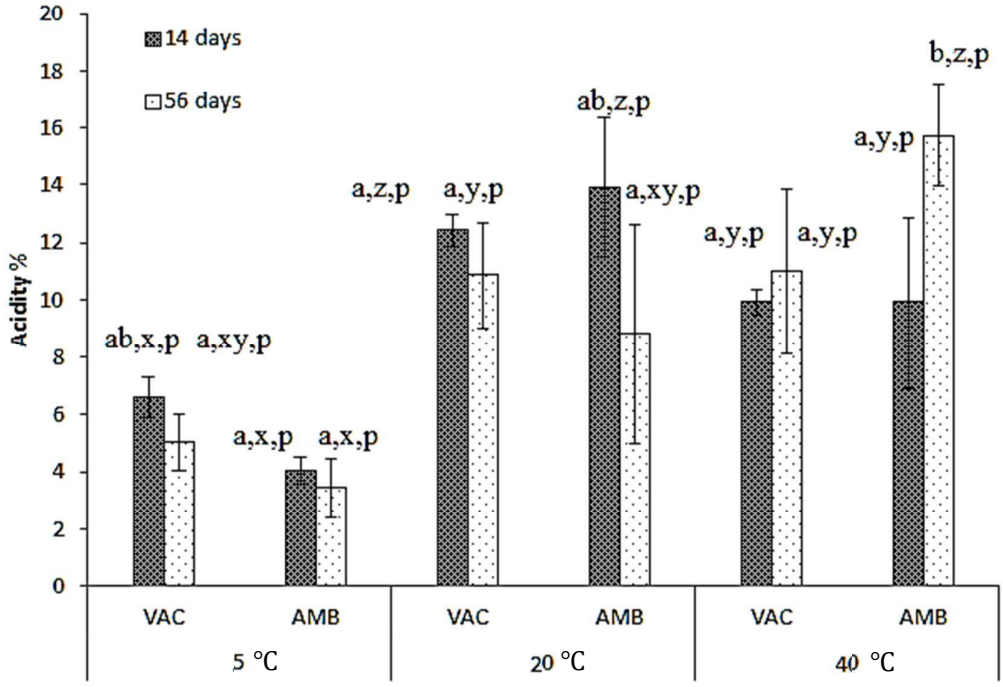

Figure 1. Free fatty acid contents (% of lipids DW; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. The values presented here were deducted from the free fatty acid content at day 0, (17.2±1.20% total lipids). For each parameter, same letters indicate similar values between storage time (a-e), temperature (w-z) and packaging conditions (p-q). Bars are representing the standard deviations, $P < 0.05$.

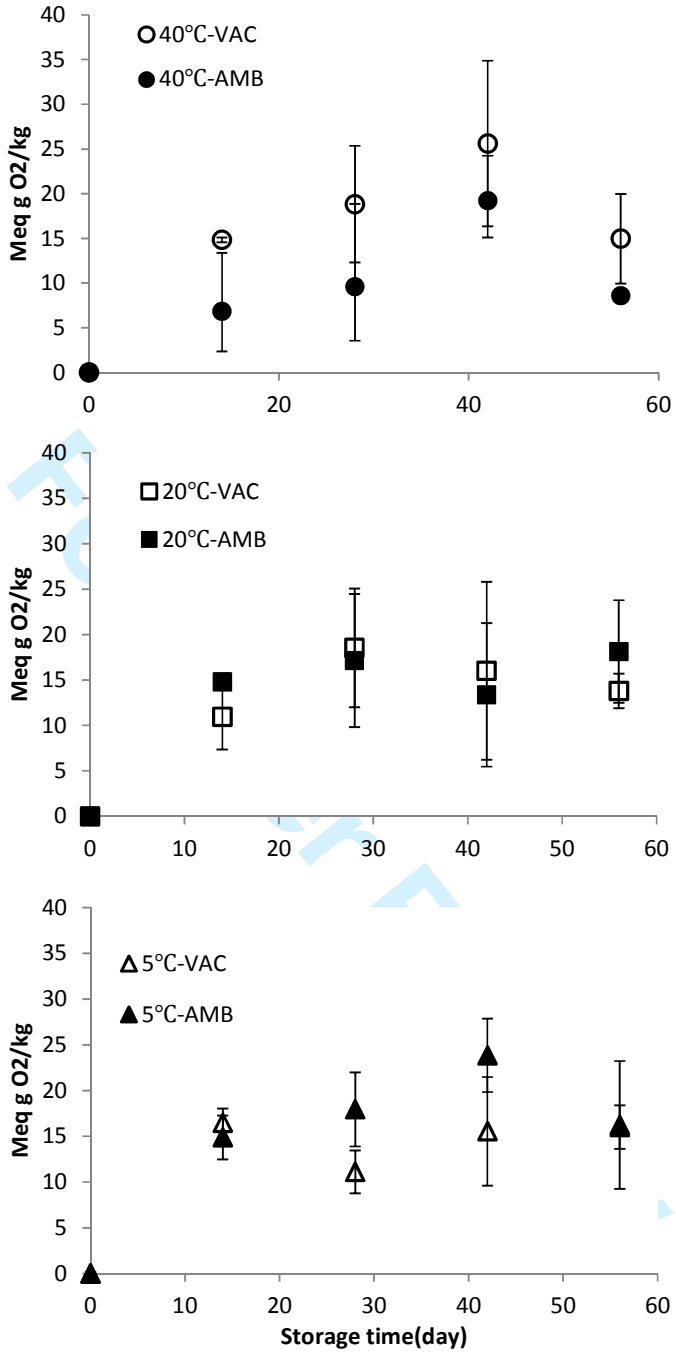

Figure 2. Absolute changes in peroxide values compared with day 0 (Meq g O2 /g lipids DW; mean ± SD; n = 4) as a function of storage time, storage temperature and packaging conditions. The values reported here were obtained by deducting the observed PV from the PV at day 0. Bars are representing the standard deviations.

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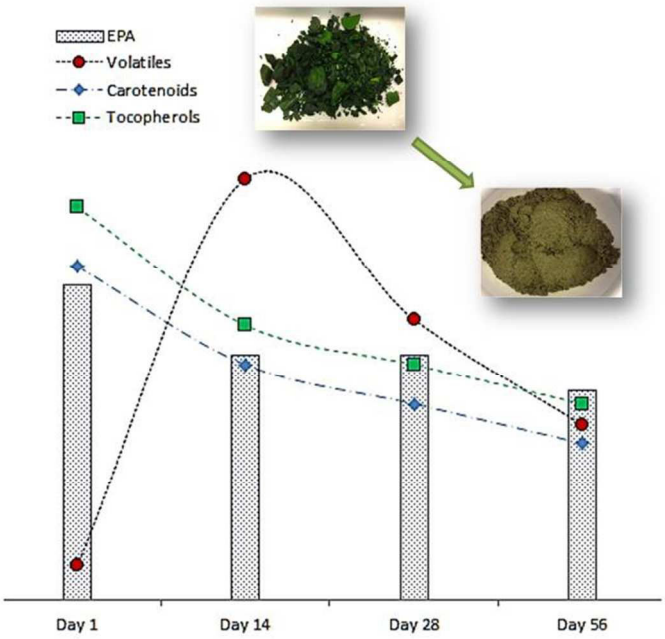

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